DEPARTAMENTO DE BIOLOGÍA MOLECULAR

FACULTAD DE CIENCIAS

UNIVERSIDAD AUTÓNOMA DE MADRID

CARACTERIZACIÓN DE LOS TUMORES DE MAMA FAMILIARES MEDIANTE CITOGENÉTICA MOLECULAR

Tesis doctoral que presenta para optar al título de

Doctor por la Universidad Autónoma de Madrid

el Licenciado en Biología

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GRUPO DE GENÉTICA HUMANA

PROGRAMA DE GENÉTICA DEL CÁNCER HUMANO

CENTRO NACIONAL DE INVESTIGACIONES ONCOLÓGICAS (CNIO)

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CERTIFICA:

Que Don Lorenzo Melchor Fernández, Licenciado en Biología por la Universidad Complutense de Madrid, ha realizado la presente Tesis Doctoral **"Caracterización de los tumores de mama familiares mediante citogenética molecular"** y que a su juicio reune plenamente todos los requisitos necesarios para optar al **Grado de Doctor en Biología**, a cuyos efectos será presentado en la Universidad Autónoma de Madrid. El trabajo ha sido realizado bajo su dirección, autorizando su presentación ante el Tribunal Calificador.

Y para que así conste se extiende el presente certificado,

Madrid, febrero 2008.

V^{o} B^{o} del Director y Tutor de la Tesis:

Javier Benítez

Esta tesis doctoral ha sido realizada en el Grupo de Genética Humana del Programa de Genética del Cáncer Humano del Centro Nacional de Investigaciones Oncológicas (CNIO), bajo la supervisión de Javier Benítez Ortiz.

Las siguientes ayudas han permitido la realización de la presente tesis doctoral:

- Ayuda de Formación de Profesorado Universitario (FPU) del Ministerio de Educación y Ciencia AP-2004-0448.
- Ayuda de corta estancia de la Unión Internacional contra el Cáncer ICRETT/05/063.
- Ayuda de corta estancia de la European Molecular Biology Organization ASTF162-05.
- Proyecto del Ministerio de Ciencia y Tecnología SAF03-02497.
- Proyecto del Ministerio de Educación y Ciencia SAF-06-06149.
- Proyecto de la Red Temática de Investigación Corporativa Centros de Cáncer C0-010.

Cuando alguien le recordó que había cosechado casi mil intentos fallidos antes de dar con el filamento de tungsteno, contestó:

"No fracasé, sólo descubrí 999 maneras de cómo no hacer una bombilla".
Thomas Alva Edison

A mis padres, Lorenzo y María Jesús
y a mi hermana Rebeca

AGRADECIMIENTOS

Tenía quince años cuando empecé a tener la ilusión de ser científico, de ponerme una bata, hacer experimentos y descubrir cosas nuevas. Transcurridos estos años de tesis, mantengo la ilusión y, aun diría más, la he renovado. Ahora escribo este apartado de la tesis reconociendo que me resulta muy difícil empezar, aunque ha sido el apartado en el que más he pensado a lo largo de estos años. Me es difícil porque representa que una etapa muy importante de mi vida profesional y sobre todo personal llega a su fin. Este es el momento de agradecer y de empezar a decir "hasta luego" a mucha gente que sinceramente no me apetece dejar de ver. Aquí van mis palabras de agradecimiento a todas y cada una de las personas que me han ayudado a lo largo de estos años:

En primer lugar, querría dar las gracias a Javier Benítez por la posibilidad que me ha brindado para realizar esta tesis. He de agradecerle la confianza que depositó en mí durante los primeros momentos que tan difíciles fueron. Creo que su paciencia ha dado al final sus frutos y que su disposición y accesibilidad para reunirse conmigo ha ayudado mucho a ello. Espero que siga haciendo crecer a este grupo de personas que conforman el Programa de Genética del Cáncer Humano del CNIO y en el que el ambiente tanto científico como social creo que puede considerarse como único.

De entre todas estas personas, quisiera comenzar por las que componen el Grupo de Citogenética Molecular. Ellas fueron mi primera familia en el CNIO acogiéndome como uno más y guiándome en el difícil mundo de los cromosomas. Recordaré a Juan Cruz Cigudosa, al que considero un ejemplo a seguir y cuyos consejos en los momentos más difíciles fueron de gran ayuda; a Sara, por sus conocimientos y por la sintonía que al final llegamos a coger; a Carmen, que fue la primera que tuvo la paciencia necesaria para enseñarme a cariotipar; a Carol, por algo que nos une más allá de nuestra afición al Getafe: la sensibilidad; y también a Bibi, Francesco, Gloria, Cristina, María, Alba y Miguel con los que tan buenos momentos he pasado. Me acordaré especialmente de Sandra y de Mamen porque nuestras tertulias y correos siempre me han hecho sonreír hasta en mis peores días y porque finalmente dimos el paso para que unas chungas del sur de Madrid y el niño de la katana pudieran conocerse, disfrutar y confiarse muchos muchos secretos, ¡gracias, chicas!.

Quisiera seguir con el Grupo de Genética Humana dando las gracias a Ana y a María, con las que me hubiera gustado aprender todavía más. Con Magda y Lara, he compartido muchas dudas pero confío en que conseguiremos lo que nos propongamos en esta vida. No olvidaré los momentos musicales con Alicia y políticos con Fernando que dan el ambiente

especial a este laboratorio. Gracias también a Fátima (¡a ver si aprendemos a tirar cervezas!), a Bárbara (cuyas frases siemrpe me descolocan) y a Eva, Toya, Bea y Gloria, pero sobre todo a Ricardo. Su alegría es contagiosa y juntos hemos pasado tantos momentos por tantos sitios tan diferentes que espero que no sean los últimos. Gracias por haberme animado siempre y porque juntos hemos descubierto "how big we are!". Te deseo todo lo mejor en tu vida y sé que sabrás disfrutarla.

Gracias también al Grupo de Cáncer Endocrino: Meme, Alberto, Rocío, Cristina Rodríguez (¡no madures!), Elena, Javi, Cristina Montero (por esos comienzos y por tu compañía en las clases de inglés), pero sobre todo a Iñigo y a Susanna. Gracias Iñigo, por tu amistad y por nuestros momentos, ya sabes que eres un tío grande, un tío fuerte, liberal, ¡vamos, de los que marcan Historia!; espero que lleguemos lejos (no sé… ¿Manhattan? ¿Ministros de Sanidad?) y sembremos de cultura muchos sitios (porque si a la gente le das cultura…) Susanna, gracias por tu paciencia a la hora de corregir mis artículos y por los euros que te tocará darme en el futuro, por los ratos de té y cañas y, sobre todo, por tu compañía y sincera amistad.

A la gente del CeGEN, en especial a Roger por sus ayudas estadísticas y en los últimos trámites de tesis, y a las que compusieron la Unidad de Cáncer Familiar: Laura, con la que compartí muchas risas, y Miguel Urioste, al que agradezco aquel día en la consulta que me permitió ver una realidad que en el laboratorio no se aprecia. Tu labor creo que es imprescindible y espero que alguna vez llegue a ser tan reconocida como se merece.

Quiero acordarme de más gente del CNIO agradeciendo la colaboración del Banco de Tumores, de la gente de Inmunohistoquímica (Raquel, Lydia, etc.) y de María Rodríguez. Gracias por los buenos ratos a esa representación internacional de Fábian, Filipe, Chaki y Lorena ("what a night!") y de Dani del Grupo de Linfomas (¡qué retreat!). A Alicía de Sistemas de Información por nuestras charlas frikis. A toda la gente del fútbol, con los que las tensiones de la semana se podían ir fácilmente con esa rivalidad entre rojos y azules. También a Ellen por sus clases de inglés, porque rescatar de mi olvido el inglés y perfeccionarlo debió de ser muy difícil.

Hay gente que ha pasado por aquí y que, de una manera u otra, me han marcado especialmente por lo que me gustaría darles las gracias: David, fuiste quien me enseñó muchas cosas en el laboratorio y te debo gran parte de lo que soy. Te guardo un gran respeto y cariño que pocos pueden entender. Sergio, ¡pijo!, generas un ambiente especial

allá donde vas, contigo todo fue mucho más fácil y nunca sabrás cuanto te he llegado a echar de menos. Emiliano, sin ti no hubiera sido posible la mitad de esta tesis y quién si no me hubiera enseñado a reconocer una célula normal de una tumoral. Y Carilla, ¿qué puedo hacer contigo? Me desmontas, macho, me desarmas por completo, no puedo parar de reír cuando estoy contigo y te agradezco especialmente aquellos inolvidables días de Pilares.

Aunque será difícil que lo lean, quiero acordarme también de aquellas personas con las que compartí momentos en mi estancia en Philadelphia. A Kate Nathanson por su confianza y colaboración, pero sobre todo a Angie, Mauri, Dilip, Erin y Pedro quiénes hicieron que aquellos meses allí fuesen dignos de grabarse en mi memoria.

Si me remonto años atrás, empecé a ver cromosomas en el Departamento de Genética de la Complutense. Por ello, quiero agradecer a mis chicas Mónica y Eva aquellas tardes de cuarto oscuro, y a Juan Luis Santos y Nieves por su guía en aquella etapa.

Quiero acordarme de mis más viejos amigos, Dani y Francisco, con los que empecé a degustar la interpretación años atrás empezando en una fría mañana de febrero. Aunque no hemos podido compartir muchos momentos durante mi tesis, he de deciros que siempre me acuerdo de vosotros. También, quiero mencionar a Ross y Javi, amigos desde la facultad, compañeros de fatigas en el CNIO y jugadores de una crónica que jamás olvidaremos. Siempre recordaré con cariño nuestras reuniones, que durante tantos años se extendieron, y todas y cada una de nuestras anécdotas juntos.

Debo agradecer además a Ross todos estos años que hemos compartido: desde aquellas clases en la facultad y sus visitas al laboratorio de genética hasta ahora. Tu compañía, consejo, amistad y cariño siempre me han servido para darme fuerzas y seguir adelante. Cristina, gracias también a ti por tu comprensión y tu infinita paciencia con nosotros.

Finalmente, quiero mencionar a aquellas personas a las que está dedicada mi tesis: a mi familia. Mi padre ha sido y es un referente a seguir de cómo afrontar los problemas y poder solucionarlos, con su guía y consejo he llegado a donde estoy. A mi madre, cuyo cariño y amor sé que son únicos y me han servido para sentirme especial en todo momento. A Rebeca, mi hermana, con la que he crecido en muchos aspectos y a la que nunca podré explicar con palabras cuanto la quiero.

En definitiva, gracias a todas estas personas y a aquellas que me han ayudado en algún momento a lo largo de todos estos años y que no he llegado a citar. Muchas gracias.

Loren

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ABREVIATURAS

A lo largo del texto de la presente tesis, el lector podrá encontrar una serie de anglicismos que hemos utilizado por su uso regular en el argot científico, si bien algunos de ellos no están admitidos en la lengua oficial en la que está escrita la tesis. A continuación se exponen las abreviaturas utilizadas en la presente tesis, algunas de las cuales proceden de anglicismos y otras son nombres de genes o proteinas:

AA	Un tumor "AA" es un tumor altamente amplificado o con un alto número de amplificaciones (>2).
aCGH	Array de CGH o array-CGH.
ADN	Ácido desoxiribonucléico.
ADNc	ADN complementario.
AECC	Asociación Española Contra el Cáncer.
AIB1	Gen coactivador 3 del receptor nuclear (nuclear receptor coactivator 3 gene).
amp	Amplificación.
Antifade	1,4 diazabiciclo-(2.2.2)-octano.
Ar-CNV	CNV Artefactual (Artifactual-CNV).
Array	Término utilizado para definir la matriz sobre la que se encuentran impresas los clones de ADN donde se realizan las hibridaciones genómicas o los cilindros tumorales donde se realizan las hibridaciones inmunohistoquímicas o valoraciones histológicas.
ARN	Ácido ribonucleico.
ARNm	ARN mensajero.
ARNt	ARN de transferencia.
ATM	Gen mutado en la ataxia telangiectasia (Ataxia Telangiectasia Mutated gene).
ATP11A	ATPasa tipo 11A (ATPase, class VI, type 11A).
BAC	Cromosoma artificial bacteriano (Bacterial Artificial Chromosome).
BACH1	Factor de transcripción con cremallera de leucina homólogo a BTB y CNC (BTB and CNC homology 1, basic leucine zipper transcription factor 1).
BARD1	Gen asociado al dominio en anillo de BRCA1 (BRCA1 associated RING domain 1 gene).
BASC	Complejo de vigilancia del genoma asociado a BRCA1.
BAX	Proteina X asociada a BCL2 (BCL2-associated X protein).
b-catenina	Catenina b.
BCLC	Consorcio para el ligamiento del cáncer de mama (Breast Cancer Linkage Consortium).
BCLXL	Proteína 1 similar a BCL2 (BCL2-like 1).
BCL2	Gen del linfoma de células B (linfocitos B) 2 (B-cell CLL/lymphoma 2 gene)
BIC	Servicio de información del cáncer de mama (Breast Cancer information Core)
BLM	Gen del síndrome de Bloom (BLoom Syndrome gene).
BPC1	Región de roturas cromosómicas 1 en el brazo 8p.
BPC2	Región de roturas cromosómicas 2 en el brazo 8p.
BRCA1	Gen de susceptibilidad al cáncer de mama 1 (Breast Cancer Gene 1).
BRCA2	Gen de susceptibilidad al cáncer de mama 2 (Breast Cancer Gene 2).
BRCAX	Tercera clase de cáncer de mama familiar no asociado a mutaciones en los genes *BRCA1* ó *BRCA2*. También catalogada como no-BRCA1/2.
BRCT	Dominio C-Terminal de BRCA1.
BRIP1	Helicasa 1 que interactúa con el dominio C-terminal de BRCA1 (BRCA1 Interacting Protein C-terminal helicase 1).

BSA	Albúmina de suero bovino (Bovine Serum Albumin).
cCGH	CGH convencional o cromosómica.
CCNA	Ciclina A.
CCNB1	Ciclina B1.
CCND1	Ciclina D1.
CCND3	Ciclina D3.
CCNE	Ciclina E.
CDH1	Caderina 1 o E-caderina (cadherin 1, type 1, E-cadherin (epithelial)).
CDH3	Caderina 3 o P-caderina (cadherin 3, type 1, P-cadherin (placental)).
CDK1	Quinasa dependiente de ciclina 1 (Cyclin-Dependent Kinase 1).
CDK2	Quinasa dependiente de ciclina 2 (Cyclin-Dependent Kinase 2).
CDK4	Quinasa dependiente de ciclina 4 (Cyclin-Dependent Kinase 4).
cen	Centrómero.
CEP	Sonda FISH centromérica.
CGH	Hibridación genómica comparativa (Comparative Genomic Hybridization).
CHK2	Gen homólogo al regulador del ciclo celular CHK2 de *S. pombe* (CHK2 checkpoint homolog (S. pombe) gene).
CK5	Citoqueratina 5 ó KRT5.
CK8	Citoqueratina 8 ó KRT8.
c-MYC	O MYC, gen homólogo al oncogén v-myc de la mielocitomatosis aviar (v-myc myelocytomatosis viral oncogene homolog (avian) gene).
CNV	Variación en el número de copias (Copy Number Variation).
CSGE	Electroforesis en gel sensible a la conformación (Conformation Sensitive Gel Electrophoresis).
CUL4A	Culina 4A.
Cy3	Cianina-3.
Cy5	Cianina-5.
dATP	2’ desoxiadenosina 5’-trifosfato.
dCTP	2’ desoxicitosina 5’-trifosfato.
del	Deleción.
DGGE	Electroforesis mediante geles con gradiente de desnaturalización.
dGTP	2’ desoxiguanosina 5’-trifosfato.
DHPLC	Cromatografía líquida desnaturalizante de alto rendimiento (Denaturing High Performance Liquid Chromatography).
dmin	Cromosomas dobles diminutos (double minute chromosomes).
DMSO	Dimetilsulfóxido.
dNTP	2’ desoinucleósido 5’-trifosfato.
DOP-PCR	PCR con cebadores de oligonucleótidos degenerados (Degenerated Oligo Primers-PCR).
dTTP	2’ desoxitimidina 5’-trifosfato.
dUTP	2’ desoxiuridina 5’-trifosfato.
EDTA	Ácido etilen diamino tetraacético.
ERBB2	Gen homólogo 2 al oncogén v-erb-B2 de la leucemia eritroblástica viral aviar (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) gene).
ESR1	Gen del receptor de estrógeno 1 (EStrogen Receptor 1 gene).
E2F6	Gen 6 de la familia de factores de transcripción E2F (E2F transcription factor 6 gene).
FAM	Fluorocromo 5-Carboxifluoresceina.
FGFR1	Receptor del factor de crecimiento fibroblástico 1 (Fibroblast Growth Factor Receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)).
FISH	Hibridación in situ de fluorescencia (Fluorescence In Situ Hybridization).

GAS6	Gen de la proteina 6 específica de detención del crecimiento (growth arrest-specific 6 gene).
Gatekeeper	Un gen *gatekeeper* es una clase de gen que regula directamente el crecimiento tumoral mediante su inhibición o activación de la muerte programada.
g-catenina	Catenina gamma o plakoglobina **(**junction plakoglobin gene).
HER2	Ver ERBB2.
HSR	Región cromosómica homogéneamente teñida (Homogeneously stained-region).
HR-CGH	CGH convencional de alta resolución (High-Resolution CGH).
IHQ	Inmunohistoquímica.
Kb	Kilobase.
Kit	Término utilizado para designar el conjunto de reactivos y material que una casa comercial determinada ofrece para realizar técnicas experimentales de uso común en el laboratorio.
Ki-67	Antigeno identificado por el anticuerpo monoclonal Ki-67 (antigen identified by monoclonal antibody Ki-67).
LA	Un tumor "LA" es un tumor levemente amplificado o con un número bajo de amplificaciones (<3).
LAMP1	Proteina lisosomal asociada a membrana (Lysosomal-Associated Membrane Protein 1).
LOH	Pérdida de heterocigosidad (Loss of Heterozigosity).
Mb	Megabase.
MDM2	Gen de la proteina que se asocia a P53 (Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) gene).
M-FISH	FISH múltiple o multicolor.
Missense	Una mutación *missense* es un tipo de mutación no sinónima en la que el cambio de un nucleótido produce un cambio de aminoácido que hace que la proteina pueda no ser funcional.
MLH1	Gen homólogo 1 a mutL de *E. coli* y causante de cáncer de colon no polipósico de tipo 2 (mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli) gene).
MRE11	Gen A homólogo al gen MRE11 de la recombinación meiótica de *S. cerevisiae* (MRE11 meiotic recombination 11 homolog A (S. cerevisiae) gene).
MSH2	Gen homólogo 2 de mutS de *E. Coli* y causante de cáncer de colon no polipósico de tipo 1 (mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli) gene).
MSH6	Gen homólogo 6 de mutS de *E. Coli* (mutS homolog 6 (E. coli) gene).
NA	Un tumor "NA" es un tumor no-amplificador o sin amplificaciones.
NBS1	Gen del síndrome de rotura cromosómica Nijmegen (Nigmegen Breakage Syndrom gene).
NER	Reparación del ADN por escisión de nucleótido (Nucleotide Excision DNA Repair process).
NFKB	(v-rel reticuloendotheliosis viral oncogene homolog A, Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells 3, p65 (avian) gene).
NHEJ	Reparación del ADN por medio de la unión de extremos no homólogos (Non-Homologous End Joining DNA repair process).
Nick Translation	Reacción de marcaje basada en el desplazamiento de la mella.
NMD	Non-sense Mediated Decay system.
Nonsense	Una mutación *nonsense* es una mutuación puntual en la secuencia del ADN que resulta en un codon de parada, que posiblemente produce una proteina truncada y a menudo no funcional.
NRG1	Neoregulina 1.
OCT	Componente utilizado para conservar el tejido a bajas temperaturas y poder realizar los cortes con un criostato de congelación (Optimal Cutting Temperature).
OOCR	Región del grupo de cáncer de ovario (Ovarian Cluster Region).
PAC	Cromosoma artifical derivado de P1 (P1 Artificial derived-Chromosome).

PALB2	Gen compañero y localizador de BRCA2 (partner and localizer of BRCA2 gene).
pb	Pares de base.
PBS	Tampón salino fosfato (Phosphate Buffered Saline buffer).
PCNA	Antígeno nuclear de la proliferación celular (Proliferating Cell Nuclear Antigen).
PCR	Reacción en cadena de la polimerasa (Polymerase Chain Reaction).
PMS1	Gen de la segregación postmeiótica incrementada (PMS1 postmeiotic segregation increased 1 (S. cerevisiae) gene).
PGR	Gen del receptor de progesterona (ProGesterone Receptor gene)
PTEN	Gen fosfatasa y homólogo de tensina (mutado en multiples cánceres avanzados) (Phosphatase and TENsin homolog –mutated in multiple advanced cancers 1— gene).
PTT	Test de la proteína truncada (Protein Truncated Test).
P120^{ctn}	Delta catenina (catenin (cadherin-associated protein), delta).
P16	Inhibidor de la ciclina quinasa 2A, también CDKN2A (cyclin-dependent kinase inhibitor 2A).
P21	Inhibidora de la ciclina quinasa 1A, también CDKN1A (cyclin-dependent kinase inhibitor 1A (p21, Cip1)).
P27	Inhibidor de la ciclina quinasa 1B, también CDKN1B (yclin-dependent kinase inhibitor 1B (p27, Kip1)).
P53	Ver TP53.
RAD50	Gen homólogo a RAD50 de *S. cerevisiae* (RAD50 homolog (S. cerevisiae) gene).
RAD51	Gen RAD51 homólogo a RecA de *E. coli*.
Ratio	Relación de dos datos.
RASA3	RAS p21 protein Activator 3.
RB	Gen del retinoblastoma.
RE	Receptor de estrógenos (ver ESR1).
RP	Receptor de progresterona (ver PGR).
RT-PCR	PCR de la transcripción reversa.
SDS	Dodecil sulfato sódico.
SKP2	Proteina 2 asociada a la quinasa de la fase S (S-phase kinase-associated protein 2 (p45)).
SKY	Cariotipo espectral (Spectral Karyotype).
SOTA	Self Organizing Tree Algorithm.
SSC	Solución salina de citrato de sodio.
SSCP	Análisis de conformación polimórfica de hebra sencilla (single-strand conformation polymorphism).
STK11	Serin-treonin quinasa 11 causante del síndrome de Peutz-Jegher (Serine/Threonine Kinase 11).
Survivina	Gen/proteina de BIRC5 (baculoviral IAP repeat-containing 5 gene).
TA	Temperatura ambiente.
TFDP1	Factor de transcripción DP-1 (Transcription Factor Dp-1).
TMA	Matriz de tejidos (Tissue MicroArray).
TOP2A o TopoII	Topoisomerasa II alfa (topoisomerase (DNA) II alpha 170kDa).
TP53	Gen de la proteina tumoral p53 (Tumor Protein p53 gene).
UNC5D	Gen D homólogo a unc-5 de *C. elegans* (unc-5 homolog D (C. elegans) gene).
UPGMA	Método de reconstrucción filogenética (Unweighted Pair Group Method with Arithmetic mean).
VHL	Gen supresor tumoral von Hippel-Lindau (Von Hippel-Lindau tumor suppressor gene).
XRCC3	Gen 3 defectuoso en la reparación del daño al AND causado por rayos X en las células de hamster chino (X-ray repair complementing defective repair in Chinese hamster cells 3 gene).

RESUMEN / ABSTRACT

El cáncer de mama familiar (~5% de los casos de cáncer de mama) puede deberse a mutaciones en los genes *BRCA1* y *BRCA2* (~30% de las familias con cáncer de mama) o a mutaciones en otros genes todavía por descubrir en las familias no-*BRCA1/2* ó BRCAX (~70%). Para caracterizar las diferencias genómicas entre las distintas clases de cáncer de mama familiar y el esporádico, hemos utilizado un total de 27 tumores de portadores de mutación en *BRCA1*, 28 tumores con mutación en *BRCA2*, 38 tumores BRCAX y 19 esporádicos. Estos tumores han sido analizadas mediante técnicas de citogenética molecular como la hibridación genómica comparativa sobre cromosomas (cCGH) y sobre arrays (aCGH). Sus resultados se pusieron en relación con variables clínicas, histológicas e inmunohistoquímicas.

Los tumores asociados a *BRCA1* y *BRCA2* presentaron una mayor inestabilidad genómica que los tumores esporádicos o BRCAX y una tendencia a alterar diferentes regiones cromosómicas. Sin embargo, hemos demostrado la existencia de una común heterogeneidad molecular entre los tumores de origen familiar y los de origen esporádico en relación al receptor de estrógenos (RE) y a la existencia de subtipos de cáncer de mama. En primer lugar, los tumores con negatividad para RE tuvieron una mayor inestabilidad genómica y un perfil de aberraciones característico en comparación con los tumores con expresión de RE, independientemente del tipo de cáncer de mama. En segundo lugar, los tumores familiares se han clasificado en varios subtipos (basal, ERBB2, luminal A y luminal B), asociándose los tumores *BRCA1* al fenotipo basal mayoritariamente y los BRCAX al luminal A. Los tumores basales presentaron una mayor inestabilidad genómica y un patrón de aberraciones característico, mientras que los tumores luminal B presentaron un mayor número de amplificaciones de alto nivel.

Además, hemos estudiado dos regiones de amplificación de alto nivel en cáncer de mama familiar: 8p11-p12 y 13q34. La primera de ellas se asoció a una mayor proliferación celular, mientras que la segunda fue una amplificación característica de tumores basales, de 1’6 Mb de tamaño y asociada a la sobreexpresión de un gen candidato (*TFDP1*).

Estos hallazgos nos han permitido proponer un modelo integrador sobre el origen y desarrollo de los distintos subtipos de tumores de mama independientemente del tipo de cáncer de mama (familiar o esporádico). Este modelo combina teorías como la hipótesis de las células troncales cancerígenas y la hipótesis de la selección clonal.

Familial breast cancer (~5% of breast cancer cases) maybe due to mutations at the two high susceptibility genes: *BRCA1* and *BRCA2* (~30% of families with breast cancer) or to mutations in other unknown genes to date in the non-*BRCA1/2* or BRCAX families (~70%). In order to characterize the genomic differences amongst the distinct breast cancer classes, we have collected 27 tumours associated with *BRCA1* mutation, 28 tumours from mutation carriers in *BRCA2*, 38 BRCAX-tumours, and 19 sporadic samples. These tumours have been analysed using molecular cytogenetics techniques such as chromosomal or array-based comparative genomic hybridization (cCGH or aCGH, respectively). The results from these analysis have been correlated with clinical, histological, and immunohistochemical features.

Breast tumours associated with mutations in *BRCA1* or *BRCA2* had greater genomic instability than BRCAX or sporadic samples, and a trend to alter specific chromosomal regions. However, we have described the existence of a common molecular heterogeneity amongst familial and sporadic breast cancer in terms of estrogen receptor (ER) status and breast cancer subtypes. First, ER-negative tumours showed higher genomic instability and a specific genomic aberration pattern compared with ER-positive tumours. Second, familial breast tumours have been profiled into molecular subtypes: basal, ERBB2, luminal A, and luminal B. *BRCA1*-tumours were associated with basal phenotype, whereas BRCAX samples were related to luminal A subtype. Basal tumours presented the highest genomic instability and the most characteristic genomic aberration pattern, while luminal B tumours had greater number of high-level DNA amplifications.

In addition, we have studied two chromosomal regions altered with high-level DNA amplifications in familial breast cancer: 8p11-p12 and 13q34. The former one was related to higher cell proliferation, whereas the latter one was characteristic of basal tumours, had 1’6Mb length, and was correlated with overexpression of candidate genes such as *TFDP1*.

These findings allowed us to propose an integrative model about the origin and development of the distinct breast cancer subtypes independently of the breast tumour type (familial or sporadic). This model combines theories such as the cancer stem cell hypothesis and the clonal selection model.

INTRODUCCIÓN

1. EL CÁNCER DE MAMA: GENERALIDADES

1.1. LA GLÁNDULA MAMARIA Y EL CÁNCER DE MAMA

La mama está formada por una serie de glándulas mamarias denominadas lóbulos y lobulillos. Tras el parto, los lobulillos secretan la leche a una red de conductillos que van drenando en conductos mayores hasta llegar al pezón. Los lóbulos y los ductos están rodeados por tejido adiposo y conectivo que, junto con el tejido linfático, terminan de constituir el seno mamario.

Las células que conforman los lóbulos y ductos se disponen en una bicapa celular: la capa interna está formada por células epiteliales secretoras (luminales), mientras que la capa externa se compone de células mioepiteliales (basales). Finalmente, una lámina basal de tejido conectivo engloba el lóbulo o conducto.

El cáncer de mama es una enfermedad compleja y heterogénea originada a partir de una proliferación anormal y desorganizada de las células que componen el tejido mamario. A grandes rasgos, el cáncer de mama se clasifica de acuerdo al componente celular principalmente afectado: carcinoma lobular o lobulillar (se afectan las células de los lóbulos o lobulillos) y carcinoma ductal (las células de los ductos). Los carcinomas que se limitan al lóbulo o conducto se denominan “carcinomas in situ”, mientras que los que se desarrollan a partir de los ductos o lóbulos y rompen la membrana basal, invadiendo tejido adiposo, conectivo y los vasos y nervios que constituyen la parte no funcional de la mama, se denominan “carcinomas invasivos o infiltrantes”.

1.2. EPIDEMIOLOGÍA

El cáncer de mama se trata de la neoplasia más frecuente entre las mujeres del mundo occidental y la principal causa de muerte entre los 35 y los 50 años. Su incidencia ha aumentado de manera progresiva desde la década de los 70 y 80, debido probablemente al envejecimiento de la población en los países desarrollados y a su diagnóstico precoz (Lacey *et al.*, 2002; Lopez-Abente *et al.*, 2000). Sin embargo, su tasa de mortalidad decrece a una estimación de 2’3% anual posiblemente gracias a una detección precoz, una mayor concienciación y un mejor tratamiento. De todas maneras, el cáncer de mama es la

primera causa de muerte por cáncer entre las mujeres. Por su parte, el cáncer de mama también se manifiesta en varones aunque con una incidencia cien veces menor.

La Asociación Española Contra el Cáncer (AECC) cifra en 370.000 los nuevos casos de cáncer de mama en la Unión Europea. En España, se diagnostican unos 16.000 nuevos casos al año y es la causa de fallecimiento de 6.000 mujeres al año (el 16'7% de las muertes por cáncer en la mujer) (Lopez-Abente *et al.*, 2003). La incidencia y la tasa de mortalidad en España son de las más bajas dentro de los países desarrollados (Figura 1A), aunque existe una variación en función de la región (Lopez-Abente *et al.*, 2003).

Por último, la incidencia de cáncer de mama aumenta de manera constante a partir de los 25 años, para estabilizarse a los 45-50 años. A partir de esta edad, se produce una disminución en los países de baja incidencia, se estabiliza en los de intermedia y sigue aumentando en los de alta incidencia (Figura 1B).

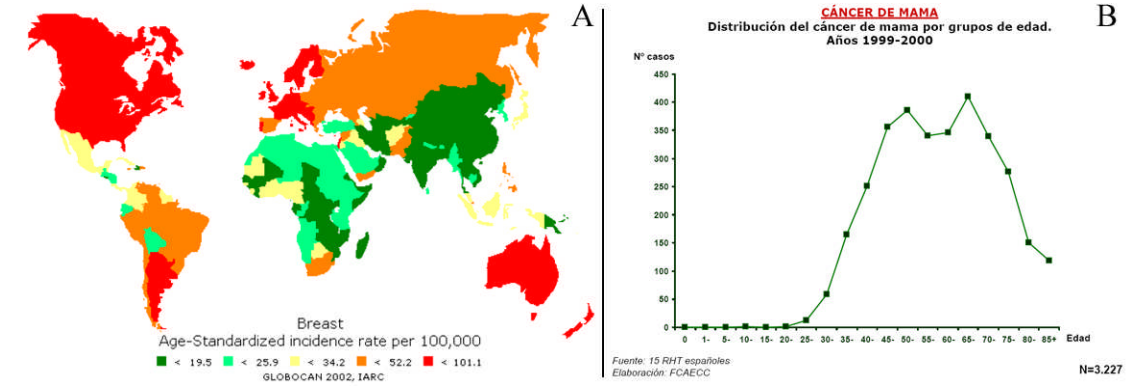


Figura 1. Incidencia del cáncer de mama. A, en todo el mundo. B, en España a lo largo de grupos de edad. Adaptado de datos de la AECC.

1.3. FACTORES DE RIESGO DE CÁNCER DE MAMA

El inicio y progreso del cáncer de mama depende de la combinación de diferentes factores exógenos (variables ambientales en las que el tiempo y periodicidad de exposición adquieren importancia) y endógenos (dependientes de la susceptibilidad genética que el individuo hereda).

Con respecto a los primeros, gran parte de los factores que incrementan el riesgo de padecer cáncer de mama están relacionados con una mayor exposición de la glándula

mamaria a los estrógenos circulantes (menarquia temprana, menopausia tardía, nuliparidad, edad tardía del primer embarazo y obesidad en mujeres posmenopáusicas). El uso de anticonceptivos orales y de terapia hormonal posmenopáusica también incrementan ligeramente el riesgo (Armstrong *et al.*, 2000; Key *et al.*, 2001). Otros factores de riesgo ambientales investigados incluyen determinadas enfermedades benignas mamarias, la edad, el sedentarismo, la exposición precoz a altas dosis de radiaciones ionizantes, el consumo de alcohol, el alto consumo de grasa, el menor consumo de folatos, la exposición a plaguicidas organoclorados, el tabaco y los campos electromagnéticos de muy baja frecuencia (Johnson-Thompson and Guthrie, 2000; Key *et al.*, 2001).

No obstante, el factor de riesgo más estudiado e importante es una historia familiar de cáncer de mama: la herencia de variables genéticas que incrementan de manera notable la probabilidad de padecer cáncer de mama. Las mujeres con alteraciones en alguno de los dos genes de alta susceptibilidad, *BRCA1* y *BRCA2*, presentan una probabilidad de desarrollar cáncer de mama a lo largo de su vida entre 60% y 85% (Armstrong *et al.*, 2000). Sin embargo, estos genes representan una pequeña proporción de los casos familiares. Mutaciones en genes como *TP53*, *ATM* o *PTEN* también incrementan el riesgo de desarrollar cáncer de mama si bien como consecuencia de un síndrome generalizado (síndromes de Li-Fraumeni, Ataxia telangiectasia o Cowden, respectivamente) (Key *et al.*, 2001).

En el caso de los varones, ciertas enfermedades como el síndrome de Klinefelter, las orquitis, cirrosis y bilharziasis, favorecen el desarrollo del cáncer de mama.

2. EL CÁNCER DE MAMA FAMILIAR-HEREDITARIO

2.1. EL CÁNCER DE MAMA FAMILIAR: DESDE LA ANTIGÜEDAD HASTA EL HALLAZGO DE GENES RESPONSABLES

Alrededor de un 5% de los casos de cáncer de mama se atribuyen a la herencia de mutaciones en genes de susceptibilidad al cáncer de mama (tipo familiar). La mayoría de los casos no exhiben un patrón claro de herencia y resultan probablemente del efecto múltiple de variables de baja penetrancia de un grupo amplio de genes que se ven, a su vez, modificados por los factores ambientales (cáncer de mama tipo esporádico). A grandes rasgos, el cáncer de mama familiar se caracteriza por una edad temprana de aparición, mayor frecuencia de casos bilaterales y la aparición de otros tipos de tumores (ovario, colon, próstata, de endometrio y sarcomas).

La primera vez que se describe la agregación de casos de cáncer de mama en una familia se remonta a la antigua Roma. Posteriormente en 1866, un cirujano francés describe 10 casos de cáncer de mama en cuatro generaciones de la familia de su mujer; en la que otras cuatro mujeres murieron de cáncer hepático (Couch and Weber, 1998). No es hasta la década de 1970 cuando se sugiere la presencia de un factor genético responsable de la incidencia inusualmente elevada de cáncer de mama en una familia con tres hermanas que presentaban cáncer de mama bilateral (Cady, 1970). En 1984, Williams y Anderson usaron análisis de segregación para comparar varios modelos que pudiesen explicar el patrón de agregación familiar de cáncer de mama (Williams and Anderson, 1984), siendo los primeros en dar una evidencia de un gen de susceptibilidad con herencia autosómica dominante. Años después, su hipótesis fue apoyada (Newman *et al.*, 1988) y demostrada cuando se localizó la región cromosómica del gen candidato (Hall *et al.*, 1990), que se identificó como *BRCA1* en 1994 (Miki *et al.*, 1994).

Los dos genes de alta susceptibilidad al cáncer de mama identificados hasta la fecha son *BRCA1* (Miki *et al.*, 1994) y *BRCA2* (Wooster *et al.*, 1995). El descubrimiento de estos dos genes ha permitido extender el conocimiento durante estos años sobre la epidemiología genética del cáncer de mama, el riesgo exacto a padecer la enfermedad, la distribución por etnias, el asesoramiento genético y las consecuencias clínicas de sus mutaciones.

2.2. *BRCA1* Y *BRCA2*: LOS GENES DE ALTA SUSCEPTIBILIDAD AL CÁNCER DE MAMA

En 1994, el grupo de Mark Skolnick clonó el gen *BRCA1* y describió diferentes mutaciones en pacientes con cáncer de mama (Miki *et al.*, 1994). Los autores corroboraron el papel de *BRCA1* como causante de la predisposición al cáncer de mama en pacientes jóvenes y con historia familiar postulado unos años antes por el grupo de Mary-Claire King mediante análisis de ligamiento (Hall *et al.*, 1990). Sin embargo, se describieron familias con una elevada incidencia de cáncer de mama en varones que no portaban mutaciones en *BRCA1* (Stratton *et al.*, 1994). Ante esto, la búsqueda de otros genes de susceptibilidad al cáncer de mama pronto dio sus frutos: en 1994, se identificó un segundo gen, *BRCA2*, ligado a la región cromosómica 13q12-q13 (Wooster *et al.*, 1994) y al año siguiente, fue clonado y caracterizado (Wooster *et al.*, 1995).

2.2.1. Mutaciones germinales y de efecto fundador

Se estima que las mutaciones en los genes *BRCA* suceden en 1 de cada 1000 mujeres. Para la detección de las mutaciones, resulta necesario seleccionar bien las familias candidatas debido a que ambos genes carecen de puntos calientes, solo un 25% de las familias presentan mutación y la complejidad y el coste de las técnicas de detección son muy elevados. Algunas técnicas de detección que se utilizan son las pruebas de proteína truncada, la cromatografía líquida desnaturalizante de alto rendimiento (DHPLC), la electroforesis mediante geles con gradiente de desnaturalización (DGGE) o la secuenciación directa.

Las mutaciones descritas para ambos genes se recopilan en una base de datos llamada “Breast Cancer Information Core” (BIC), establecida en 1995 para ir registrando el rango y frecuencia de mutaciones germinales de *BRCA1* y *BRCA2* (Couch and Weber, 1996). El 87% de las mutaciones que se describen de *BRCA1* causan la ausencia de expresión o la producción de una versión truncada de la proteína. Esto se debe a pequeñas inserciones o deleciones en la secuencia genética o a mutaciones *nonsense*, que introducen codones de terminación (Castilla *et al.*, 1994; Friedman *et al.*, 1994; Miki *et al.*, 1994; Simard *et al.*, 1994). Algo similar sucede con *BRCA2* (Couch *et al.*, 1996; Lancaster *et al.*, 1996; Miki *et al.*, 1996; Neuhausen *et al.*, 1996; Phelan *et al.*, 1996; Tavtigian *et al.*, 1996;

Wooster *et al.*, 1995). En ambos genes mutados, las proteínas que se sintetizan son versiones truncadas y, por lo tanto, no funcionales.

Existen mutaciones específicas que aparecen con una frecuencia elevada en ciertos grupos étnicos y reciben el nombre de mutaciones fundadoras. La población judía es un claro ejemplo ya que, de presentar mutación en los genes *BRCA*, es altamente probable que presenten alguna de las siguientes mutaciones: 185delAG o 5382insC en *BRCA1* (Simard *et al.*, 1994), o 6174delT en *BRCA2* (Oddoux *et al.*, 1996). Otros ejemplos de mutaciones fundadoras se presentan en las poblaciones de Islandia (Tulinius *et al.*, 2002), Polonia (Gorski *et al.*, 2004) y España (Diez *et al.*, 2003). La importancia de las mutaciones de efecto fundador radica en la posibilidad de dirigir las pruebas genéticas hacia estas mutaciones en aquellas pacientes de cáncer de mama familiar de una etnia determinada.

Otra clase de mutaciones menos frecuente (~13%) son las mutaciones *missense* (de cambio de aminoácido), las cuales son difíciles de distinguir de polimorfismos neutrales en ausencia de ensayos funcionales.

2.2.2. La penetrancia de las mutaciones en *BRCA1* y *BRCA2*

Ser portador de una mutación no implica que definitivamente se desarrolle la enfermedad. Este hecho es un aspecto fundamental en el asesoramiento genético y ha de tenerse especialmente en cuenta antes de ofrecer opciones preventivas drásticas como la mastectomía profiláctica. La penetrancia es el riesgo a lo largo de la vida de desarrollar cáncer de mama u ovario y habitualmente el riesgo se limita hasta los 70 años. Las portadoras de mutación en *BRCA1* tienen un riesgo de 60-80% de desarrollar cáncer de mama (Easton *et al.*, 1993), de 20-40% de cáncer de ovario (Easton *et al.*, 1995), así como de otros cánceres como próstata y colon (Ford *et al.*, 1994). Por su parte, las mutaciones en *BRCA2* también tienen una penetrancia del 60-85% de cáncer de mama. A diferencia de *BRCA1*, los varones con mutación en *BRCA2* tienen un riesgo de cáncer de mama (6%) cien veces superior a la población control. El riesgo de cáncer de ovario en portadoras de mutación en *BRCA2* es menor (10-20%). Las mutaciones en *BRCA2* también se asocian a un incremento en el riesgo de otros cánceres como próstata, colon, páncreas, etc. (Easton and BCL, 1999; Edwards *et al.*, 2003). La penetrancia puede verse afectada por diversos parámetros:

- Localización y tipo de la mutación: se ha visto que mutaciones en la región central de *BRCA1* pueden asociarse a un menor riesgo de cáncer de mama (Scott *et al.*, 2003). El cambio a codon de terminación 6174delT de *BRCA2*, típico de población judía, tiene una baja penetrancia (28% en lugar del habitual 80% de *BRCA2*) (Warner *et al.*, 1999). Por otro lado, las mutaciones de cambio de aminoácido (*missense*) 300T>G en *BRCA1* y 4486G>T en *BRCA2* se asocian a un mayor riesgo de cáncer de mama (Scott *et al.*, 2003).
- Factores genéticos: la existencia de variantes en los genes modificadores como *TP53* (Osorio *et al.*, 2006) ó *RAD51* (Levy-Lahad *et al.*, 2001; Wang *et al.*, 2001).
- Factores ambientales: como el uso de anticonceptivos orales, ooforectomía y el número de partos (Narod, 2002), o el estilo de vida como el ejercicio físico o la ausencia de obesidad en la adolescencia que retrasan el desarrollo de cáncer de mama (King *et al.*, 2003).

2.2.3. Estructura de los genes *BRCA1* y *BRCA2*

El gen *BRCA1* se localiza en 17q21. Está compuesto de 24 exones, que transcriben un ARN mensajero de 7.173 pb de longitud (Figura 2A). Del total de exones, 22 de ellos codifican una proteína de 1.863 aminoácidos. La estructura de *BRCA1* es inusual ya que el tamaño medio de los exones es de 140 pares de bases, excepto el del exón 11, de aproximadamente 3.500 pb, que constituye casi el 60% de la región codificante. La proteína BRCA1 posee varios dominios funcionales por los que interacciona con multitud de proteínas y con el ADN (Figura 2B). Cabrían destacar el dominio en forma de anillo ("RING finger domain"), situado cerca del extremo N-terminal, y el dominio BRCT (BRCA1 C-Terminal) compuesto de secuencias repetitivas claves para la interacción con proteínas implicadas en la reparación del ADN o en el metabolismo.

Por su parte, *BRCA2* se localiza en 13q12-q13. Mayor que *BRCA1*, tiene un total de 10.930 nucleótidos distribuidos en 27 exones, de los que 26 son traducidos produciendo una proteína de 3.418 aminoácidos (Figura 2C). Al igual que *BRCA1*, también presenta un exón 11 muy grande, con un tamaño de 5.000 pb. La proteína BRCA2 solo contiene dos dominios funcionales conocidos de los que destacan las repeticiones BRC (similares al

BRCT de BRCA1) situadas en la región central de la proteína y que son esenciales para la función de BRCA2 en la reparación del ADN y la interacción con RAD51 (Figura 2D).

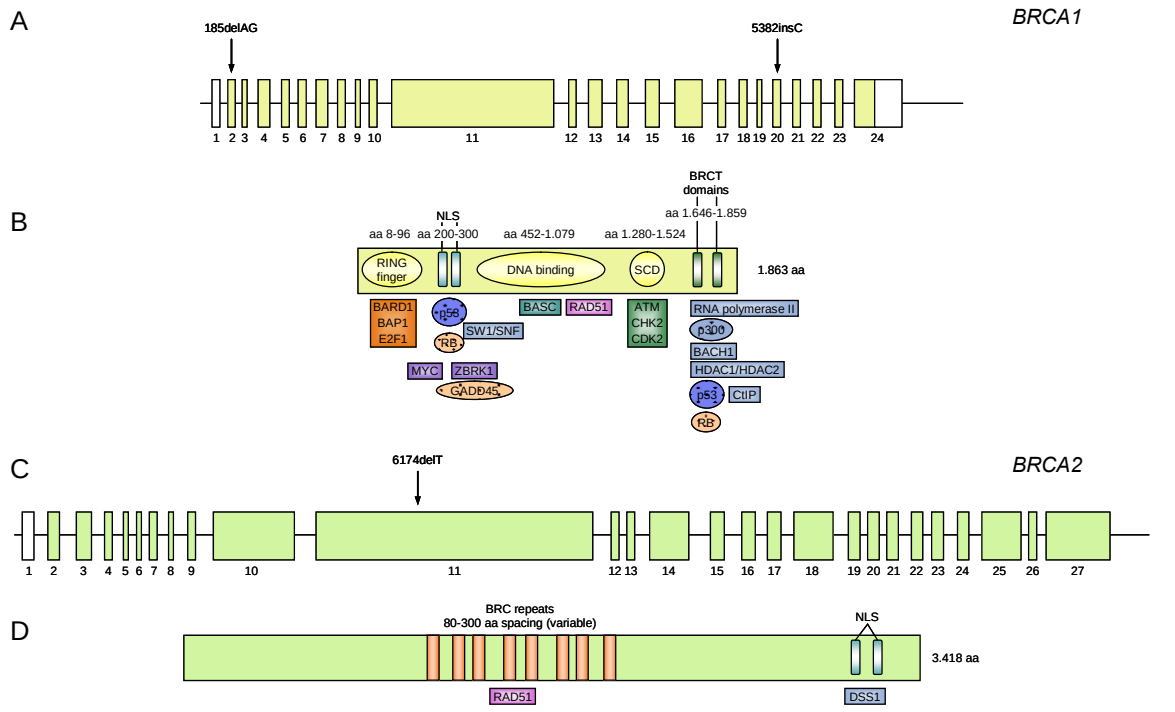


Figura 2. Representaciones de BRCA1 y BRCA2. Esquemas del gen BRCA1 (2A) y BRCA2 (2C) y sus proteínas (2B, 2D) en las que se destacan los diferentes dominios de interacción con proteínas. Adaptado de (Narod and Foulkes, 2004)

2.2.4. Funciones de los genes *BRCA1* y *BRCA2*

BRCA1 y *BRCA2* son considerados genes "gatekeepers", cuya mutación o expresión alterada provoca que la célula pierda los controles normales de la división y muerte programada, promoviendo el crecimiento de las células cancerosas. Ambos genes se involucran en la reparación del ADN, pero se sabe que *BRCA1* participa además en los controles del ciclo celular, de la ubiquitinación de proteínas y de la remodelación de la cromatina.

Reparación del ADN

BRCA1 y BRCA2 participan en complejos proteicos que activan la reparación de roturas de doble hebra e inician la recombinación homóloga. Tras el daño en el ADN, ATM desencadena una serie de reacciones que culminan en la fosforilación de BRCA1 mediada por CHK2 (Cortez *et al.*, 1999; Zhang *et al.*, 2004). Una vez fosforilada, BRCA1 es capaz de interaccionar con BARD1, RAD51 y BRCA2 para participar en la

recombinación homóloga. En este proceso, RAD51 juega un papel clave al asociarse con BRCA2, a través de las repeticiones BRC y de su dominio C-terminal (Wong *et al.*, 1997). Una vez asociadas, se sitúan en las regiones de daño del ADN junto con BRCA1 formando los complejos de reparación del ADN (Chen *et al.*, 1998a; Scully *et al.*, 1997).

El único sistema de reparación libre de errores es la recombinación homóloga. Cuando esta vía no funciona correctamente, las roturas de doble cadena son reparadas por un mecanismo propenso a cometer errores, como la unión de extremos no homólogos ("non-homologous end joining", NHEJ), y los errores pueden conducir a que se produzcan reordenamientos cromosómicos (Patel *et al.*, 1998; Zhong *et al.*, 1999). Esta inestabilidad cromosómica resultante representa uno de los rasgos claves de la carcinogénesis. BRCA1 actúa en un doble papel como activador de la reparación por recombinación homóloga e inhibidor de la reparación por unión de extremos no homólogos (Zhang *et al.*, 2004). Se ha visto que las células que presentan mutaciones en *BRCA1* o en *BRCA2* son más sensibles a las radiaciones ionizantes y, por ello, más propensas a cometer errores durante la reparación. Asimismo, también son muy sensibles a agentes que producen roturas en la doble cadena de ADN, como el cisplatino y la mitomicina C (Moynahan *et al.*, 2001; Tassone *et al.*, 2003; Yuan *et al.*, 1999).

BRCA1 también participa en la reparación por escisión de nucleótido (NER) a través de los mecanismos de la reparación acoplada a transcripción (Le Page *et al.*, 2000) y de la reparación genómica global (Hartman and Ford, 2002).

En resumen, se podría concluir que BRCA1 y BRCA2 actúan en una ruta común que es responsable de la integridad del genoma y del mantenimiento de la estabilidad cromosómica (Venkitaraman, 2002).

Control del ciclo celular

Los puntos de control son necesarios para comprobar si existen errores durante el ciclo celular. En caso de encontrar un error, el ciclo se detiene para permitir que la célula pueda subsanarlo. Si no se puede reparar el error, entra en funcionamiento la maquinaria apoptótica que conducirá a la célula hacia la muerte celular programada. En estos puntos de control, BRCA1 interviene a través del complejo proteico BASC (complejo de vigilancia del genoma asociado a BRCA1) (Wang *et al.*, 2000). Otras proteínas forman

parte de este complejo como NBS1 (gen del síndrome de rotura Nijmegen), RAD50-MRE11, ATM (cuyo gen está alterado en la ataxia telangiectasia), BLM (síndrome de Bloom), el factor C de replicación del ADN y los complejos MLH1-PMS1 y MSH2-MSH6.

Los modelos murinos *Brca1* -/- ó *Brca2* -/- mueren durante los primeros momentos de la embriogénesis (Hakem *et al.*, 1996; Xu *et al.*, 1999). La letalidad embrionaria de estos ratones puede verse retrasada durante algunos días ante una pérdida de p53 ó p21 (Hakem *et al.*, 1997). El mal funcionamiento del punto de control del ciclo celular puede ser un paso crucial en la carcinogénesis. La mayoría de las células que carecen de los genes *BRCA1* y *BRCA2* experimentan apoptosis gracias a los puntos de control. Sin embargo, si proteínas cruciales de los puntos de control tales como p53 o p21 están alteradas, las células sobreviven sufriendo una inestabilidad genómica que les llevará a poseer importantes anomalías en el cariotipo.

Ubiquitinación

El proceso por el cual las proteínas son "marcadas" para ser degradadas se denomina ubiquitinación. Las proteínas involucradas en este proceso poseen un dominio en forma de anillo a través del cual interactúan. Un claro ejemplo son BRCA1 y BARD1 que, con el dominio en anillo cerca del extremo N-terminal, participan en la ubiquitinación (Wu *et al.*, 1996). Recientemente se ha descrito que la ubiquitinación mediada por BRCA1 ocurre en respuesta al daño en el ADN (Morris and Solomon, 2004).

Remodelación de la cromatina

Varios complejos proteicos se implican en remodelar la cromatina en las cercanías de los sitios de rotura de la doble hebra de ADN para favorecer la reparación. En este sentido, BRCA1 forma parte del complejo BASC, que actúa en este proceso, pero también interacciona con el complejo SW1-SNF, con histonas desacetilasas o con BACH1 para la remodelación de la cromatina y una correcta reparación.

Otras funciones

Se han descrito papeles de BRCA1 en el silenciamiento del cromosoma X o como regulador de la transcripción de varios genes (Narod and Foulkes, 2004; Zheng *et al.*, 2000).

2.2.5. Las alteraciones somáticas en los genes *BRCA1* y *BRCA2*

Genes que se implican en cáncer hereditario como *RB* (Retinoblastoma) o *VHL* (Von Hippel-Lindau) aparecen mutados con frecuencia en los tumores esporádicos correspondientes (Blanquet *et al.*, 1995; Gnarra *et al.*, 1994). Del mismo modo, se postuló que *BRCA1* y *BRCA2* podrían relacionarse con el desarrollo de los tumores de mama en general. Sin embargo, varios autores buscaron mutaciones somáticas en ambos genes sin éxito alguno en cáncer de mama esporádico (Futreal *et al.*, 1994; Lancaster *et al.*, 1996; Teng *et al.*, 1996).

A pesar de ello, sí se han descrito otros mecanismos de inactivación de *BRCA1* en tumores de mama esporádicos. La metilación aberrante del promotor de *BRCA1* se encuentra en el 11-14% de los cánceres de mama esporádicos y en el 5-31% de los cánceres de ovario (Catteau *et al.*, 1999; Esteller *et al.*, 2000). Esta inactivación a menudo se acompaña de la pérdida del otro alelo de *BRCA1* o pérdida de heterocigosidad (LOH) (Esteller *et al.*, 2000; Staff *et al.*, 2003), lo que lleva a que un alelo se haya perdido por deleción y el restante no se exprese por la hipermetilación de su promotor. Otros medios de inactivación de *BRCA1* que acarrean una menor expresión proteica son: la pérdida de una copia alélica que podría ocasionar haploinsuficiencia (Staff *et al.*, 2003), la sobreexpresión de inhibidores de la expresión de *BRCA1* (Turner *et al.*, 2007), u otros mecanismos todavía por conocer (Magdinier *et al.*, 1998).

2.3. FAMILIAS DE RIESGO

De todas las mujeres con cáncer de mama en la familia, solo unas pocas poseen una historia familiar que sugiera realmente que un gen de alta penetrancia como *BRCA1* ó *BRCA2* sea el responsable de la susceptibilidad. En este sentido, se propusieron una serie de criterios para dividir las familias con cáncer de mama en dos grupos (Hoskins *et al.*, 1995):

2.3.1. Familias de alto riesgo

Caracterizadas por la presencia de cáncer de mama en parientes cercanos (al menos tres casos), la enfermedad parece seguir un modelo de herencia autosómico dominante

(Claus *et al.*, 1991; Claus *et al.*, 1990; Easton *et al.*, 1993). Otras características son la edad media temprana de diagnóstico de cáncer de mama (inferior a 50 años) y la aparición de casos de cáncer de ovario y otros tumores (Easton *et al.*, 1993; Narod *et al.*, 1995). La predisposición al cáncer en estas familias es resultado de una mutación en un gen de susceptibilidad de alta penetrancia, como es el caso de los genes *BRCA1* y *BRCA2*.

2.3.2. Familias de riesgo moderado

Caracterizadas por la presencia de dos mujeres con cáncer de mama en la familia, ausencia de cáncer de ovario y una edad media de diagnóstico avanzada (Claus *et al.*, 1991; Claus *et al.*, 1990). Se desconoce la base genética de estas familias, pero probablemente no se deba a la herencia de un gen de susceptibilidad de alta penetrancia, sino más bien se ajustaría a un modelo de herencia de varios genes de baja penetrancia.

A partir de esta primera clasificación, existen distintos criterios de selección más o menos estrictos. En cualquier caso, hay unanimidad en que la implicación de *BRCA1* y *BRCA2* está por lo general restringida a las familias de alto riesgo.

2.4. OTROS GENES DE SUSCEPTIBILIDAD CONOCIDOS Y LAS FAMILIAS BRCAX

En un principio se estimó que las mutaciones en *BRCA1* y *BRCA2* eran causantes del incremento de la susceptibilidad a padecer cáncer de mama en el 75% de las familias con cáncer de mama y en la mayoría de las familias con cáncer de mama y ovario (Easton *et al.*, 1993; Ford *et al.*, 1994). Sin embargo, las estimaciones más recientes apuntan a que solo serían responsables de un 25% de las familias, lo cual se mantiene en cualquiera de las poblaciones analizadas (Diez *et al.*, 2003; Hakansson *et al.*, 1997; Meindl, 2002; Osorio *et al.*, 2000; Serova *et al.*, 1997; Shih *et al.*, 2002).

Como hemos mencionado anteriormente, existen genes implicados en síndromes de cáncer hereditario en los que se registra un considerable aumento en el riesgo de padecer cáncer de mama: *TP53*, causante del síndrome de Li-Fraumeni (Malkin *et al.*, 1990); *ATM*, responsable de la ataxia telangiectasia (Bishop and Hopper, 1997); *PTEN*, responsable del síndrome de Cowden (Tsou *et al.*, 1997); *STK11/LKB1*, causante del síndrome de Peutz-Jegher (Boardman *et al.*, 1998), etc. Sin embargo, todos estos genes solo representan una

pequeña proporción de las familias con cáncer de mama y ninguno puede considerarse como *BRCAX*.

La búsqueda de un gen *BRCAX* de susceptibilidad al cáncer de mama puede realizarse mediante dos metodologías: análisis de ligamiento y estudios de asociación.

2.4.1. Análisis de ligamiento

Trabajos recientes mediante análisis de ligamiento han propuesto la existencia de genes candidatos de susceptibilidad al cáncer de mama en varios loci: 8p12-p22 (Seitz *et al.*, 1997), 13q21 (Kainu *et al.*, 2000), 2q32 (Huusko *et al.*, 2004) y 10q23.32-q25.3 (Bergman *et al.*, 2007). Nuestro grupo también ha contribuido en este campo recientemente proponiendo seis regiones candidatas diferentes a las ya propuestas, remarcando así la heterogeneidad que existe en el grupo BRCAX (Gonzalez-Neira *et al.*, 2007). Sin embargo, se requiere más trabajo en este área para delimitar los genes candidatos y caracterizarlos apropiadamente.

2.4.2. Estudios de asociación

Uno de los problemas en la búsqueda de genes de baja penetrancia radica en que las familias en las que se encuentran mutados dichos genes no presentan un número suficiente de casos que pueda usarse en los estudios tradicionales de ligamiento. Por consiguiente, los estudios de asociación se han postulado como un mejor procedimiento mediante el análisis de genes de susceptibilidad al cáncer de mama de baja penetrancia que se suponen, a su vez, como modificadores de genes de alta penetrancia. En este sentido, *CHEK2* se considera un gen de baja penetrancia en el que la mutación 1100delC confiere un riesgo levemente elevado de cáncer de mama en europeos (3’1% en pacientes con historia familiar de cáncer de mama) (Vahteristo *et al.*, 2002) y americanos (5’1% de los pacientes con historia familiar de cáncer de mama sin mutación en *BRCA1/2*) (Meijers-Heijboer *et al.*, 2002). El predominio de esta mutación tiene una gran variación regional, siendo común en el norte de Europa (Países Bajos y Finlandia), raro entre americanos del norte (Offit *et al.*, 2003; Oldenburg *et al.*, 2003) o nulo en población mediterránea (Caligo *et al.*, 2004; Osorio *et al.*, 2004). Por otro lado, otros dos nuevos genes, asociados a subtipos concretos de la anemia de Fanconi, han sido descritos como genes de susceptibilidad al cáncer de mama de baja penetrancia: *BRIP1* (riesgo relativo de 2’0) (Seal *et al.*, 2006) y *PALB2*

(riesgo relativo de 2’3) (Rahman *et al.*, 2007). La lista de otros genes modificadores de *BRCA1/2* incluye al receptor de andrógenos, receptor de progesterona, *AIB1*, entre otros (Nathanson and Weber, 2001); pero, a pesar de todos los esfuerzos realizados, el descubrimiento de un tercer gen de alta penetrancia para cáncer de mama (*BRCA3*) sigue resultando esquivo a los investigadores.

3. CARACTERIZACIÓN CITOGENÉTICA DEL CÁNCER DE MAMA

Como se ha mencionado al inicio de esta introducción, el cáncer de mama es una enfermedad compleja y heterogénea. Con el objetivo de estudiar esta heterogeneidad, definir asociaciones con el pronóstico y mejorar los actuales tratamientos, los investigadores han tratado de caracterizar el cáncer de mama mediante diversos métodos. Dado que el objetivo central de esta tesis es el estudio del cáncer de mama familiar mediante técnicas de citogenética molecular, dedicaremos este apartado a presentar los principales hallazgos descritos hasta el momento en este campo.

3.1. DE LA CITOGENÉTICA CONVENCIONAL A LA MOLECULAR

El interés por las aberraciones cromosómicas en las células tumorales tiene su inicio en 1914, cuando Theodore Boveri postula, en su edición alemana de "*El origen de los tumores malignos"*, que el crecimiento tumoral se basa en "una combinación de cromosomas incorrecta, causante de un crecimiento anormal y transmitida a las células hijas" (Boveri, 1914). A partir de entonces, el desarrollo de las técnicas de citogenética fue crucial en el avance de los conocimientos sobre las alteraciones cromosómicas presentes en las células tumorales. Entre esas técnicas se puede contar el cariotipo convencional mediante bandeo cromosómico, la hibridación *in situ* de fluorescencia (FISH) y sus variantes (M-FISH, SKY, etc.), y la hibridación genómica comparativa sobre cromosomas (CGH convencional o cromosómica, cCGH) o su versión más reciente sobre matrices de clones (*array-CGH*, o aCGH) (Albertson *et al.*, 2003). Por su parte, las aberraciones cromosómicas que pueden describirse van desde las alteraciones de tipo numérico (ganancias o pérdidas de cromosomas o regiones cromosómicas) hasta las alteraciones de tipo estructural (intercambios de material cromosómico entre cromosomas o translocaciones). Entre las primeras, es importante remarcar la posible ganancia o amplificación de regiones que contienen genes potencialmente oncogénicos y la mutación de genes o pérdida genómica de regiones que contienen genes con función supresora del crecimiento tumoral.

El perfil de aberraciones cromosómicas presentes en el genoma tumoral engloba tanto el número y tipo de aberración como las regiones cromosómicas que se ven

recurrentemente alteradas. Este perfil varía bastante en función del tumor estudiado (mama, colon, leucemia, etc.) y, a su vez, de los diferentes tipos de un tumor (en el caso de tumores de mama: familiar/esporádico, ductal/lobulillar, invasivo/*in situ*, etc.).

3.1.1. La citogenética convencional y los primeros estudios moleculares

Los estudios de citogenética convencional de pacientes con cáncer de mama son numerosos desde la década de 1950, si bien la mayoría se basan en cariotipos sin bandeo cromosómico (Sandberg, 1990) (Figura 3A). Los análisis citogenéticos con bandeo cromosómico se debatieron entre el estudio directo del material tumoral o de las líneas celulares establecidas a partir de tumores de mama (Figura 3B). No obstante, varios autores coincidieron en que los cromosomas que se alteraban de manera recurrente en el cáncer de mama eran, de mayor a menor frecuencia: 1, 11, 3, 6 y 17 (Ferti-Passantonopoulou and Panani, 1987; Gebhart *et al.*, 1986; Gebhart *et al.*, 1984; Kovacs, 1978; Rodgers *et al.*, 1984; Trent, 1985). Estos cromosomas se describían habitualmente afectados por reordenamientos, pero las trisomías y tetrasomías también se describían en los cromosomas 3, 7 y 19.

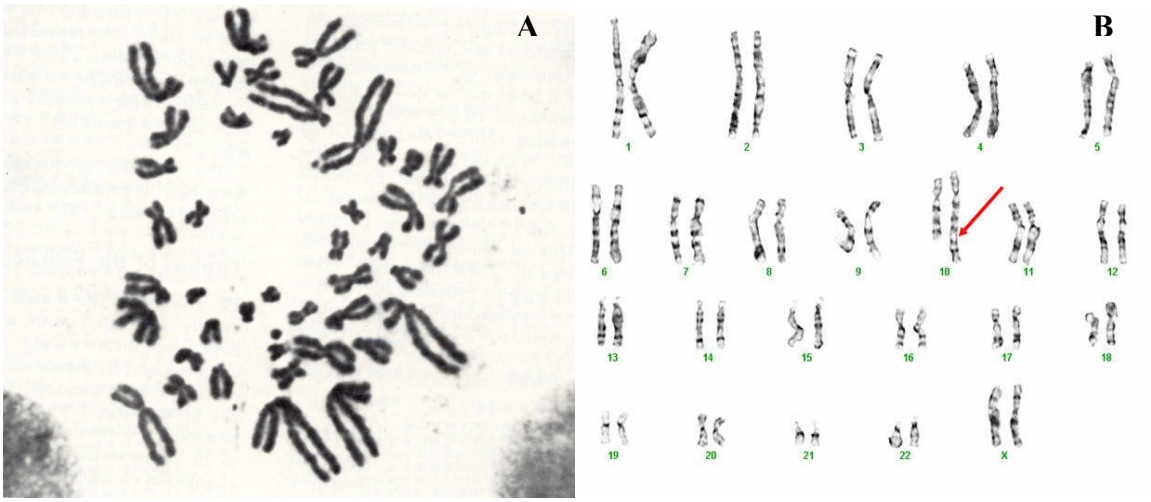


Figura 3. Ejemplos de citogenética convencional. A, ejemplo de una metafase sin bandear de la efusión pleural de un paciente con cáncer de mama. Se observan al menos seis cromosomas anormales. Extraído de (Sandberg, 1990). B. Cariotipo con bandas G de un caso de cáncer de mama. Se señala un exceso de material en el brazo largo del cromosoma 10.

Los estudios de Gebhart y colaboradores se centraron además en la descripción de los equivalentes citogenéticos de la amplificación génica: los cromosomas diminutos o *dmins* ("double minutes") y las regiones homogéneamente teñidas o *HSR* ("homogeneously stained region") (Gebhart *et al.*, 1986; Gebhart *et al.*, 1984). Con estos trabajos, quedó demostrado que las amplificaciones se presentaban en un alto porcentaje de

tumores de mama y que su presencia a menudo se asociaba a un peor pronóstico, mostrando con ello que los hallazgos citogenéticos podrían servir de valor pronóstico en carcinomas o tumores sólidos además de en leucemias.

Los primeros estudios moleculares confirmaron las amplificaciones de diferentes oncogenes y su asociación con un peor pronóstico y/o el desarrollo tumoral como por ejemplo *ERBB2* (Fontaine *et al.*, 1988; Slamon *et al.*, 1989) y *C-MYC* (Escot *et al.*, 1986; Kozbor and Croce, 1984). También se analizaron las frecuentes pérdidas de heterocigosidad (LOH) en los cromosomas 13 (Lundberg *et al.*, 1987) y 11 (Ali *et al.*, 1987).

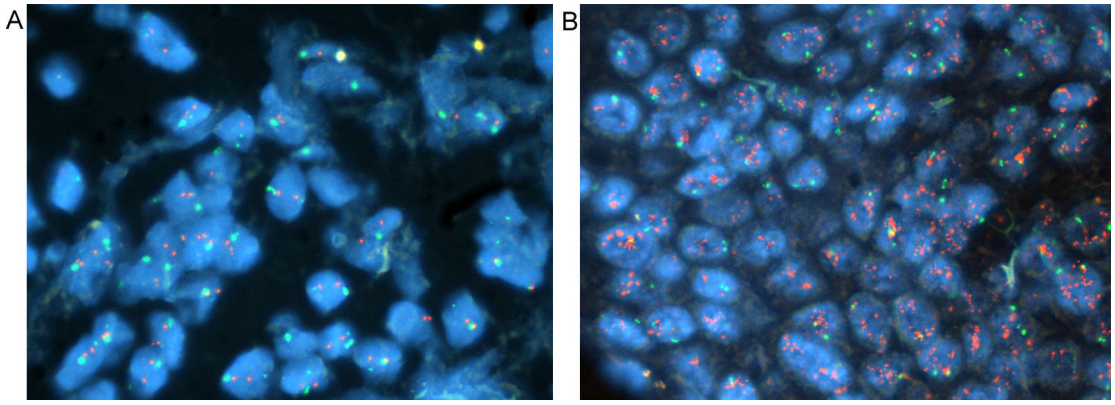


Figura 4. Ejemplo de FISH con sonda ERBB2 en muestras parafinadas. Las sondas verdes señalan el centrómero del cromosoma 17, mientras que las señales rojas hibridan en la región del gen ERBB2. A, muestra tumoral negativa para la amplificación dado que se ven 2 señales rojas y 2 verdes. B, tumor positivo para la amplificación con numerosas señales rojas y 2 verdes por célula.

3.1.2. Los primeros avances en citogenética molecular: la FISH

La citogenética molecular ha sido de particular importancia en el estudio del cáncer, ya que además del número de cromosomas o las alteraciones en éstos, ha permitido obtener mucha más información sobre el genoma de las células estudiadas. Una de las técnicas con mayor importancia y aplicación en la clínica es la Hibridación *In Situ* con Fluorescencia (FISH, del inglés "Fluorescent in situ hybridization") que comenzó a desarrollarse a mediados de la década de 1980 (Cremer *et al.*, 1986; Pinkel *et al.*, 1986). Esta técnica permite el análisis de núcleos en interfase y el estudio detallado de dosis génica (amplificaciones o deleciones) y reordenamientos estructurales (translocaciones e inversiones) con el diseño apropiado de sondas. La aplicación de la FISH más utilizada en la clínica del cáncer de mama es la detección de la amplificación de *ERBB2* en secciones de parafina (Hicks and Tubbs, 2005), permitiendo la identificación de pacientes que

podrían beneficiarse del tratamiento con el anticuerpo Trastuzumab. Un ejemplo se muestra en la Figura 4.

3.1.3. Hacia un estudio global del genoma: la Hibridación Genómica Comparativa

Si bien técnicas aplicadas de la FISH como el cariotipo espectral (SKY) o la FISH múltiple (M-FISH) se usan para un estudio global de todos los cromosomas humanos, marcados cada uno en un color diferente y en una sola hibridación, el verdadero salto en la citogenética molecular se produce con el desarrollo de una técnica que permite el estudio de todo el genoma simultáneamente en busca de pérdidas o ganancias de material cromosómico. La dificultad en la obtención de metafases a partir de tumores sólidos representaba un problema al que hacer frente para poder abordar una caracterización genómica más detallada de los tumores sólidos en general.

En 1992, se desarrolló la técnica de la Hibridación Genómica Comparativa (CGH, del inglés "Comparative Genomic Hybridization") sobre cromosomas (Kallioniemi *et al.*, 1992). Esta técnica se basa en el aislamiento del ADN genómico de una muestra control y de otra muestra a estudiar, su posterior fragmentación y marcaje con dos fluorocromos distintos para una hibridación sobre un cristal en el que hay cromosomas normales en metafase (Ver Material y Métodos epígrafe 4, para una mejor descripción). La CGH permitiría el análisis del genoma tumoral en busca de ganancias y pérdidas de material cromosómico con la premisa de que cambios en la dosis génica, causado por aberraciones en el número de copias, contribuyan probablemente al desarrollo y progresión del cáncer. Sin embargo, los reordenamientos estructurales no pueden ser caracterizados usando esta técnica.

El primer estudio de CGH en cáncer de mama data de 1994 (Kallioniemi *et al.*, 1994a). Los autores compararon la inestabilidad genómica presente en una serie de líneas celulares y de muestras de cáncer de mama, describieron las regiones que se veían frecuentemente afectadas con ganancias o amplificaciones (1q, 8q, 17q22-q24 y 20q12-q13) y validaron los hallazgos con el uso de la FISH. A partir de entonces se sucedieron numerosas publicaciones sobre cáncer de mama basadas en CGH tales como la optimización de la técnica con muestras fijadas en parafina (Isola *et al.*, 1994), el estudio

de regiones de amplificación (Tanner *et al.*, 1994), o los primeros análisis de tumores seleccionados por pronóstico (Isola *et al.*, 1995), metástasis (Kuukasjarvi *et al.*, 1997), presencia de HSR (Muleris *et al.*, 1995) o por patrón de ploidía del DNA (Ried *et al.*, 1995). Un ejemplo de una cCGH se muestra en la Figura 5.

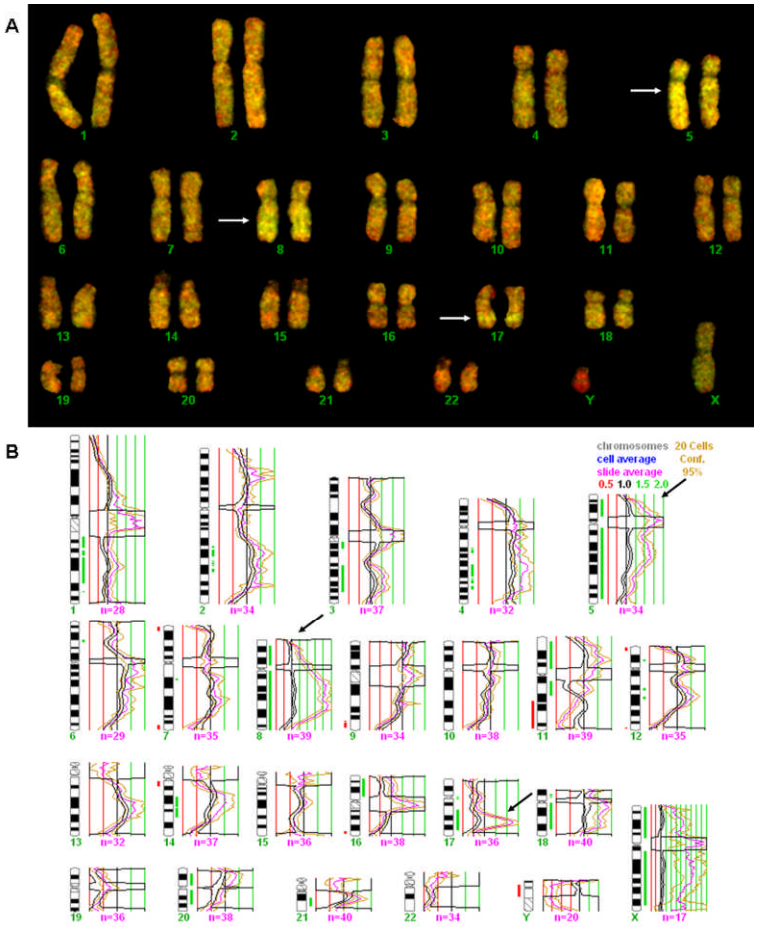


Figura 5. Ejemplo de cCGH de una muestra en parafina. A, cariotipo de cCGH en el que se señalan una serie de cromosomas con regiones con un mayor número de copias genómicas (cromosomas 5, 8 y 17). B, perfil de cCGH donde se muestran las ganancias (barras verdes a la derecha del idiograma) y las pérdidas (barras rojas a la izquierda del idiograma), asimismo se muestran los perfiles de los ratios a lo largo de los cromosomas, indicando los diferentes niveles de ganancias. Las flechas negras señalan los perfiles de las regiones indicadas en la figura A, mostrando así el mayor número de copias del cromosoma 5 y 8 y la amplificación de alto nivel de una región del cromosoma 17. Destaca la ganancia genómica del cromosoma X (en verde en la figura A) y la pérdida genómica del cromosoma Y (rojo en la figura A), debido a que la hibridación se ha realizado enfrentando mujer (marcado en verde) y varón (marcado en rojo) por lo que el doble número de copias del cromosoma X en la mujer marca en verde el cromosoma X de esta metafase normal y el ADN procedente del cromosoma Y (marcado en rojo) es lo único que hibrida con el cromosoma Y de esta metafase normal.

3.1.4. El camino hacia una mayor resolución: La matriz o array de CGH

Sin embargo, una de las grandes limitaciones de la técnica de CGH convencional o cromosómica (cCGH) es su relativa baja resolución. Esto se debe a que la definición de las aberraciones genómicas y de sus límites se reduce a una escala citogenética (~10 Mb). Por ello, el avance de la citogenética molecular más destacado lo representa la técnica de la hibridación genómica comparativa sobre matrices, o *array* de CGH (aCGH). En esta técnica, la hibridación se realiza sobre un cristal en el que están impresos cientos e incluso miles de clones que representan desde regiones concretas del genoma a la totalidad de éste. La naturaleza de los clones es variada y así lo demuestran las diferentes plataformas que surgieron aproximadamente hace una década: BACs (de "Bacterial Artificial Chromosomes"), P1, cósmidos o de ADNc (Pinkel *et al.*, 1998; Pollack *et al.*, 1999; Snijders *et al.*, 2001; Solinas-Toldo *et al.*, 1997). La versatilidad en el diseño de la matriz de CGH permite hacer estudios de: a) regiones concretas del genoma como los subtelómeros (Veltman *et al.*, 2002); b) el análisis detallado de cromosomas concretos (Kauraniemi *et al.*, 2001; Pinkel *et al.*, 1998; Ray *et al.*, 2004; Roylance *et al.*, 2006), o c) todo el genoma mediante el uso de 2.500-4.500 BACs dispuestos a una resolución de ~1Mb (Greshock *et al.*, 2004; Snijders *et al.*, 2001) o de 32.433 BACs solapados sin dejar espacios sin cubrir (Ishkanian *et al.*, 2004). El aCGH permite así relacionar la aberración genómica no solo con la región cromosómica (como la cCGH hacía) sino que también con la secuencia genómica, por lo que se podrían identificar qué genes se encuentran posiblemente afectados en el tumor (Figura 6). Los análisis de aCGH están ayudando de manera considerable en la búsqueda de nuevos marcadores pronósticos, en la identificación de nuevos oncogenes o genes supresores de tumores y, en último término, en una mejor comprensión de los procesos de formación y evolución de los tumores.

3.2. LA CARACTERIZACIÓN DE REGIONES DE AMPLIFICACIONES DE ALTO NIVEL

Las primeras aplicaciones de las técnicas avanzadas de citogenética molecular (cCGH y aCGH) fueron encaminadas a la caracterización de regiones en el genoma alteradas con amplificaciones de alto nivel ("amplicones"). Este procedimiento se basaba en la premisa de que en esas regiones podrían estar localizados genes que al estar en un mayor número de copias se sobreexpresarían de manera que podrían favorecer el origen y desarrollo tumoral, y por tanto considerarse como oncogenes o proto-oncogenes. Por lo tanto, el

interés de estos estudios radica en el posible valor pronóstico que representaría el tener o no un determinado amplicón y, a su vez, el desarrollo de tratamientos concretos, como el trastuzumab ante la amplificación de *ERBB2*, que favorecería la respuesta al tratamiento (Figura 6).

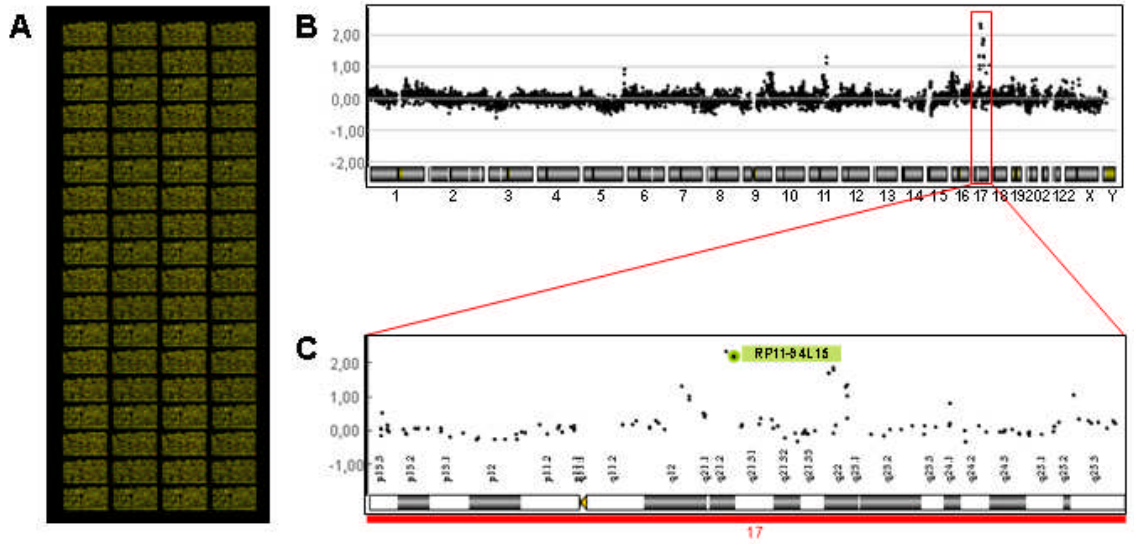


Figura 6. Análisis de array de CGH. A, ejemplo de un array de CGH de BACs. B, patrón de aberraciones genómicas de un tumor, donde el eje de coordenadas refleja los cromosomas humanos y el eje de abscisas los valores de log2ratio del clon en el array. C, patrón de aberración del cromosoma 17 en el que se destaca el clon RP11-94L15 como altamente amplificado. Este clon solapa con el gen ERBB2, lo que pone de manifiesto la relación de la aCGH no solo con la región cromosómica sino que también con la secuencia genómica.

En el año 2000, un estudio global de cCGH de 38 líneas celulares de cáncer de mama describió amplificaciones de alto nivel que contenían oncogenes conocidos tales como 8q24 (*MYC*), 11q13 (*CCND1/INT2/EMS1*), 17q12 (*ERBB2*) y 8p11-p12 (*FGFR1*) (Forozan et al., 2000). En este estudio, se cita también una serie de amplificaciones recurrentes de regiones cromosómicas en las que no se conocía la existencia de ningún proto-oncogén relacionado con cáncer de mama como por ejemplo 8q23, 3q25-q26, 17q22-q24, 1p13, 1q32, 5p13, 5p14 y 7q21-q22 (Forozan et al., 2000). La definición de los amplicones más recurrentes y la búsqueda de otros genes distintos a los conocidos que pudieran favorecer también el desarrollo tumoral ha sido centro de muchos estudios. Algunas de estas regiones fueron por ejemplo: 17q12 (Kauraniemi et al., 2001) y 8p11-p12 (Garcia *et al.*, 2005; Gelsi-Boyer *et al.*, 2005; Prentice *et al.*, 2005; Ray *et al.*, 2004). Por su parte, los trabajos en pos de una definición de aquellos amplicones en los que no se conocía ningún proto-oncogén candidato han sido prolíficos y objetos de discusiones en la comunidad científica. Algunos ejemplos son: 17q22-q24 (Barlund *et al.*, 2000; Barlund *et*

al., 1997; Monni *et al.*, 2001; Wu *et al.*, 2001) ó 20q13 (Albertson *et al.*, 2000; Collins *et al.*, 2001; Pinkel *et al.*, 1998; Tanner *et al.*, 1994).

La mayoría de los estudios de definición de amplicones se han realizado con líneas celulares o muestras de cáncer de mama esporádico. En lo que respecta al cáncer de mama familiar, solo Tirkkonen y cols. describieron levemente las amplificaciones en 17q22-q24 y 20q13 como más frecuentes en tumores *BRCA2* (Tirkkonen et al., 1997), por lo que hay cierto desconocimiento de la frecuencia, localización e importancia de los amplicones en el cáncer de mama familiar.

3.3. LOS PERFILES DE ABERRACIONES GENÓMICAS EN EL CÁNCER DE MAMA MEDIANTE cCGH Y aCGH

3.3.1. La inestabilidad genómica en el cáncer de mama esporádico

El laboratorio de Olli P. Kallioniemi fue el primero en publicar un estudio de cCGH detallado del patrón de cambios genómicos en una colección de tumores primarios de mama que no habían sido previamente seleccionados. En su trabajo, describieron como aberraciones frecuentes las ganancias de 1q (67%), 8q (49%), 16p (38%) y 5p (24%) o las pérdidas de 8p (29%), 13q (25%), 17p (22%). Sugirieron además que las ganancias de 1q son eventos genéticos tempranos en el desarrollo tumoral, mientras que las ganancias de 8q se producen en tumores más avanzados (Tirkkonen *et al.*, 1998). A partir de este estudio, se sucedieron muchos otros que profundizaron en la caracterización del cáncer de mama esporádico a través de dos procedimientos:

a) La caracterización citogenética de tipos tumorales previamente establecidos. Roylance y colaboradores describieron las diferencias existentes entre tumores de alto y de bajo grado: en los primeros existe una mayor inestabilidad genómica, mientras que en los segundos hay una asociación característica con la pérdida de 16q que sugiere que los tumores de bajo grado no evolucionan hacia tumores de alto grado (Roylance *et al.*, 1999). Por otra parte, el tipo histológico también ha servido para diferenciar tumores y, a partir de su caracterización citogenética, hipotetizar diferentes vías de evolución tumoral (Buerger *et al.*, 1999a; Buerger *et al.*, 1999b).

b) Clasificación de los tumores en función de sus aberraciones genómicas. Algunos trabajos han clasificado los tumores en función de las regiones que presentaban amplificación y, a su vez, se han visto asociados a diferentes características como al estado del receptor de estrógenos o al tipo histológico (Courjal *et al.*, 1997; Courjal and Theillet, 1997). Rennstam y cols. definieron tres grupos en función de sus aberraciones genómicas y, a su vez, asociaron estos grupos con un peor/mejor pronóstico y otra serie de variables como grado histológico, tamaño tumoral, estado de los receptores de estrógenos y progesterona (Rennstam *et al.*, 2003). También existen trabajos que estudian las aberraciones genómicas y postulan modelos de evolución en los que dichas alteraciones van adquiriéndose (Hoglund *et al.*, 2002; Molist *et al.*, 2004).

Los primeros estudios con aCGH del cáncer de mama esporádico son muy recientes (Loo *et al.*, 2004; Nessling *et al.*, 2005; Nyante *et al.*, 2004). En el primer trabajo, se pusieron de manifiesto las diferencias citogenéticas entre tumores lobulillares, ductales, invasivos e *in situ* (Nyante *et al.*, 2004). Loo y cols. asociaron dos patrones de aberraciones genómicas diferentes en base al estado de los receptores de estrógenos y al tipo histológico en 44 tumores (Loo *et al.*, 2004). Finalmente, el estudio de Nessling y cols describió el patrón genómico de 31 tumores de pacientes de cáncer de mama avanzado con al menos 10 ganglios axilares afectos (Nessling *et al.*, 2005).

3.3.2. Los tumores *BRCA1* y *BRCA2* y la construcción de clasificadores

La primera caracterización genómica de los tumores de origen hereditario describió que los tumores asociados a mutaciones germinales en *BRCA1* ó *BRCA2* presentaban mayor inestabilidad genómica que los tumores esporádicos. Se describió una serie de regiones alteradas tanto en casos esporádicos como hereditarios (p.ej. +1q, +8q, +16p, -8p), pero también una serie de aberraciones genómicas asociadas a *BRCA1* (-5q, -4q, -4p, -2q, -12q) o a *BRCA2* (-13q, -6q, +17q22-q24 y 20q13). De este modo, se postuló que la acumulación de cambios genómicos durante la progresión tumoral podría seguir una única ruta en aquellos individuos genéticamente predispuestos a padecer cáncer, y especialmente aquéllos con *BRCA1* mutado (Tirkkonen *et al.*, 1997) (Tabla 1).

Con el transcurso de los años, se intentó desarrollar una aplicación de la cCGH en la clínica. Dado que existen una serie de aberraciones genómicas más frecuentes en los tumores hereditarios, la caracterización de un tumor mediante cCGH nos podría clasificar este tumor como asociado o no asociado a mutaciones en los genes *BRCA1/2*. De este modo, si a tenor de las aberraciones genómicas presentes en un tumor, éste se considera asociado a *BRCA1*, el siguiente paso es realizar una búsqueda de mutaciones en este gen. En esta línea, el laboratorio de Petra Nederlof desarrolló y perfeccionó un clasificador para tumores *BRCA1* (van Beers *et al.*, 2005; Wessels *et al.*, 2002). Otro predictor-clasificador fue diseñado por nuestro grupo basándose en la combinación de los cambios genómicos más significativos, que tenían lugar en seis regiones cromosómicas, con el estado del receptor de estrógenos. En este estudio, un conjunto considerable de casos BRCAX se clasificaban como *BRCA1* y parte de los mismos presentaban una metilación aberrante del promotor de *BRCA1* (Alvarez *et al.*, 2005) (Tabla 1).

Tabla 1. Principales estudios de cCGH con cáncer de mama familiar

Estudio (Autor y año)	Tipo de estudio	Muestras (número)	Regiones significativamente asociadas a una clase de cáncer de mama
Tirkkonen *et al,* 1997	Caracterización citogenética global de tumores *BRCA1*, *BRCA2* y esporádicos	*BRCA1* (21) *BRCA2* (15) Esporádicos (55)	-2q, -4p, -4q, -5q, -12q, -13q, +6p, +10p, +17q, -1p, -2q, -3p, -6q, -11q, -13q, +17q22-q24, +20q13 -16q
Wessels *et al*, 2002	Construcción de un clasificador *BRCA1* con tumores *BRCA1* y esporádicos, 84% de exactitud.	*BRCA1* (28) Esporádicos (42)	-3p, -4p, -5q, -12q, -16p, -18q, +3q, +7p, +8q, +10p, +12p +16p, +17q
van Beers *et al*, 2005	Construcción de un clasificador con tumores *BRCA1*, *BRCA2* y esporádicos. 83% de exactitud en un clasificador *BRCA1*, pero la exactitud decrece en los *BRCA2*. Postulan que *BRCA2* es muy semejante a esporádicos.	*BRCA1* (36) *BRCA2* (25) Esporádicos (30)	-1p13-pcen, -3ptel-p22, -3q13-q27, -9p, -10p12-pcen, -10p12-q21, -13q, -18p, +5cen-q23, +12q21-q24, +14q -3q13-q27, -9q22-q34
Álvarez *et al*, 2005	Construcción de un clasificador usando *BRCA1* y *BRCA2*. 76,7% de exactitud. Prueba en un conjunto de muestras BRCAX.	*BRCA1* (15) *BRCA2* (13) BRCAX (34)	-15q22-q26, -18p, -18q -2p11-p21, +8p11-p12, +12q11-q21

Las leves discrepancias que existen entre estos estudios en relación a las regiones que se asociaban a cada uno de los grupos podrían atribuirse al diferente trasfondo genético de las poblaciones estudiadas (de Islandia, Holanda y España) o al número de muestras analizadas en cada serie. No obstante, estos estudios ponían de manifiesto la posible existencia de diferentes rutas genéticas de evolución en cada uno de los grupos de cáncer de mama, especialmente en los tumores *BRCA1*.

3.3.3. El estudio de la heterogeneidad genómica de los tumores BRCAX

La citogenética molecular del grupo de tumores familiares sin mutación en los genes *BRCA1/2* ha sido poco estudiada hasta el momento (Alvarez *et al.*, 2005; Gronwald *et al.*, 2005; Hedenfalk *et al.*, 2003) y algunos de los estudios han ido encaminados hacia la búsqueda de un gen candidato (Kainu *et al.*, 2000; Maguire *et al.*, 2005). En lo que respecta a su caracterización citogenética, cabe destacar el trabajo del grupo de Jeffrey Trent que describió la heterogeneidad presente en un conjunto de 16 tumores BRCAX con análisis de expresión génica y dotación genómica usando la misma plataforma: dos grupos fueron descritos con perfiles de expresión y aberraciones genómicas diferentes y, en el segundo de ellos, la amplificación en 8q24 era bastante frecuente (Hedenfalk et al., 2003). Por otra parte, esta heterogeneidad quedó también patente en un estudio realizado por nuestro grupo en el que un conjunto de casos BRCAX presentaban un patrón genómico muy similar al de tumores *BRCA1*, de los que se vio que una gran mayoría tenían hipermetilación del promotor de *BRCA1* (Alvarez *et al.*, 2005). Sin embargo, más estudios son necesarios en este terreno para ahondar en la descripción de esta clase de tumores.

4. CARACTERIZACIÓN DEL CÁNCER DE MAMA POR OTRAS TÉCNICAS DE ANÁLISIS MASIVO

Si en el anterior epígrafe de esta tesis nos centrábamos en el análisis de las variaciones en el contenido de ADN en los tumores mamarios, dedicaremos en esta sección unas líneas a resumir los hallazgos más interesantes con otras técnicas de análisis masivo centradas en la variación del contenido de ARN-mensajero (los arrays de expresión) o de la cantidad de proteínas producidas (matrices de tejidos o *tissue-arrays*) en el cáncer de mama.

4.1. ANÁLISIS DE EXPRESIÓN GÉNICA: LA HETEROGENEIDAD MOLECULAR DEL CÁNCER DE MAMA

Los llamados "arrays de expresión" se diferencian de los arrays de CGH en que lo que está impreso en el cristal es ADNc, esto es ARNm que se retrotranscribe produciendo la secuencia de ADN de un gen solo con sus exones. Al hibridar ADNc procedente de un tumor con un control, se estudian las variaciones en los niveles de expresión de un conjunto amplio de genes.

Los primeros estudios de la expresión de los tumores de mama demostraron la heterogeneidad molecular dentro del cáncer de mama. Con una lista de 534 genes, se logró definir cinco grupos con unos patrones de expresión diferentes: el subtipo de cáncer tipo basal, el subtipo asociado a sobreexpresión de ERBB2, el subtipo de cáncer de mama similar a tejido normal y los subtipos luminales A y B (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003). Es interesante destacar que el subtipo luminal A se asoció a un mejor pronóstico, mientras que el subtipo basal y ERBB2 lo hacían a un peor pronóstico (Sorlie et al., 2001).

La tecnología de los arrays de expresión nació con el objetivo de servir en la práctica clínica de modo que, ante un perfil de expresión dado de un tumor, éste se asociaría con un pronóstico concreto y con un tratamiento personalizado. En este sentido, el estudio de Laura Van't Veer es el más reconocido al describir una firma de 70 genes capaz de clasificar los tumores en buen y mal pronóstico (van 't Veer et al., 2002). Esta firma de genes ya está comercializada en un chip y es de uso diario en la clínica en los Estados Unidos (Mammaprint®).

El cáncer de mama familiar ha sido también objeto de estudio con esta tecnología. Van't Veer y cols. describieron una firma genética capaz de clasificar tumores *BRCA1* y de diferenciarlos de los esporádicos con un estado negativo de los receptores de estrógenos (van 't Veer et al., 2002). Estos mismos tumores *BRCA1* son analizados por Sorlie *et al*, quiénes los asocian con el subtipo basal, mientras que los tumores *BRCA2* los relacionan con el subtipo luminal A (Sorlie et al., 2003). Años antes, un análisis de 7 tumores *BRCA1* y de 8 tumores *BRCA2* ya mostraba variación en los niveles de expresión de 176 genes, proponiendo que estas diferencias moleculares deberían estudiarse en detalle (Hedenfalk et al., 2001). Los tumores BRCAX también fueron analizados y se distinguieron dos grupos con perfiles de expresión totalmente diferentes (Hedenfalk et al., 2003). Sin embargo, se necesitan más estudios con un mayor número de muestras para profundizar en las características de los tumores familiares.

4.2. CARACTERÍSTICAS HISTOPATOLÓGICAS E INMUNO-HISTOQUÍMICAS DEL CÁNCER DE MAMA FAMILIAR

El gran tamaño de los genes *BRCA1/2* hace que el estudio de los mismos sea un proceso largo y costoso. Este hecho, sumado a que solamente el 25% de las familias estudiadas van a tener una mutación en *BRCA1/2*, ha llevado a algunos grupos a intentar caracterizar desde un punto de vista morfológico e inmunohistoquímico estas mutaciones con el fin de facilitar la selección de pacientes candidatos a la prueba genética a partir del análisis del tumor y no de los antecedentes familiares. Los estudios del Consorcio de Ligamiento del Cáncer de Mama ("Breast Cancer Linkage Consortium") han demostrado que el cáncer que se presenta en portadores de mutación en los genes *BRCA1* y *BRCA2* se diferencian morfológicamente del cáncer de mama esporádico de edad comparable (Lakhani *et al.*, 1997; Lakhani *et al.*, 1998; Lakhani *et al.*, 2002).

Los tumores de portadores de mutación en *BRCA1* presentan una alta incidencia de carcinoma medular y un alto grado histológico (Lakhani et al., 1997). En cuanto al estudio inmunohistoquímico (IHQ), los tumores *BRCA1* se han caracterizado por una alta frecuencia de casos negativos para receptores de estrógenos (RE) y progesterona (RP) (Armes *et al.*, 1998; Foulkes *et al.*, 2004b; Lakhani *et al.*, 2002; Palacios *et al.*, 2003), por sobreexpresión de P53 (Crook *et al.*, 1998; Lakhani *et al.*, 2002; Lynch *et al.*, 1998;

Palacios *et al.*, 2003) y poca o inexistente expresión de ERBB2 (Armes *et al.*, 1999; Lakhani *et al.*, 2002; Palacios *et al.*, 2005a).

Por otro lado, los tumores de portadores de mutación en *BRCA2* no muestran ninguna diferencia significativa en el tipo histológico comparando con esporádicos (Lakhani *et al.*, 1997; Lakhani *et al.*, 1998), aunque hay estudios que los relacionan con carcinomas lobulillares (Armes *et al.*, 1998; Marcus *et al.*, 1997). Tienden a ser de un grado más alto que controles esporádicos, aunque esta asociación no es tan fuerte como en los casos *BRCA1* (Lakhani *et al.*, 1997). Los tumores *BRCA2* presentan positividad para RE y RP (Armes *et al.*, 1999; Lakhani *et al.*, 2002; Palacios *et al.*, 2003), variable expresión de TP53 y ERBB2 (Armes *et al.*, 1999; Lakhani *et al.*, 2002; Palacios *et al.*, 2005a) y una tinción citoplásmica de RAD51 (Honrado et al., 2005b).

Finalmente, los tumores BRCAX han sido poco estudiados. En general, presentan un menor grado histológico que los tumores asociados a *BRCA1/2* y tumores esporádicos (Lakhani *et al.*, 2000; Palacios *et al.*, 2003). Desde el punto de vista de su IHQ, los tumores BRCAX presentan un porcentaje de casos positivos alto para RE y RP y leve para ERBB2, mientras que mayoritariamente son negativos para p53 (Palacios *et al.*, 2003).

Hay que resaltar que a lo largo de los años de desarrollo de esta tesis, nuestro grupo ha profundizado en paralelo en el estudio de la IHQ de los tumores familiares, con el aumento en el número de marcadores analizados y en la definición de grupos con un perfil IHQ concreto semejante al obtenido en los análisis de expresión.

OBJETIVOS

El cáncer de mama familiar es una enfermedad poco frecuente y cuya caracterización molecular está siendo desarrollada en los últimos años. Nuestro laboratorio ha implantado varias líneas de investigación para pormenorizar en las características moleculares de cada una de las clases de cáncer de mama familiar (asociado a mutación en *BRCA1*, asociado a mutación en *BRCA2* y el no asociado a mutaciones en *BRCA1/2* ó BRCAX). De acuerdo a la literatura presente en la fecha de inicio de esta tesis, la caracterización genómica de los tumores familiares se había desarrollado en un bajo número de estudios y utilizando técnicas de hibridación genómica comparativa sobre cromosomas (cCGH). Por lo tanto, nuestros objetivos en el inicio de esta tesis fueron los siguientes:

1) Caracterización genómica del cáncer de mama familiar mediante técnicas de citogenética molecular de alta resolución: cCGH de alta resolución (HR-CGH) y array de CGH de 1 Mb (aCGH).

   a. Descripción de aberraciones genómicas comunes a todas las clases de cáncer de mama familiar y esporádico, así como de aquellas regiones que pudieran ser específicas de alguna clase.

   b. Descripción del patrón de aberraciones genómicas específico de tumores *BRCA1* y de tumores *BRCA2* para la construcción y/o validación de clasificadores.

   c. Descripción molecular detallada de la clase BRCAX.

2) Caracterización genómica de regiones de amplificación de alto nivel de ADN en cáncer de mama familiar para la búsqueda de oncogenes que puedan representar posibles dianas terapéuticas.

3) Relación de los hallazgos obtenidos mediante los estudios citogenéticos con los resultados procedentes de la caracterización inmunohistoquímica y de los datos clínicos obtenidos por nuestro grupo en paralelo.

MATERIAL Y MÉTODOS

1. PACIENTES Y MUESTRAS ESTUDIADAS

Los pacientes de cáncer de mama familiar procedían de tres centros en España: el Centro Nacional de Investigaciones Oncológicas (CNIO), la Fundación Jiménez Díaz en Madrid y el Hospital Santa Creu i Sant Pau en Barcelona. Todos los pacientes fueron seleccionados por pertenecer a familias de alto riesgo genético. Estas familias presentaban al menos tres miembros afectados con cáncer de mama/ovario y al menos uno de ellos menor de 50 años de edad siguiendo los criterios previamente establecidos. El caso índice de cada familia fue estudiado para las mutaciones en los genes *BRCA1* y *BRCA2* por una combinación de las técnicas de SSCP, CSGE y PTT. Algunos de estos resultados han sido publicados previamente (Diez *et al.*, 2003; Osorio *et al.*, 2000). Siempre que fue posible, se obtuvo una muestra del tumor del paciente para estudios morfológicos, inmunohistoquímicos y moleculares.

1.1. SERIES DE MUESTRAS PARA LOS DIFERENTES ESTUDIOS

A lo largo de la presente tesis, hemos desarrollado un total de cinco estudios en los que el número de muestras ha ido variando en función de la disponibilidad de tejido tumoral, de la calidad del ADN extraído y del tipo de estudio. En la Tabla S 1, desglosamos en mayor detalle las muestras utilizadas en estos estudios, con sus características de tipo de mutación, edad, grado histológico, estado del receptor de estrógenos y subtipo de cáncer de mama, así como los estudios en los que se han incluido.

Primer estudio: usamos un total de 80 tumores para un análisis de la acumulación de amplificaciones empleando la técnica de cCGH. Los tumores se repartían en 26 muestras de portadores de mutación en *BRCA1*, 18 de portadores *BRCA2* y 36 muestras BRCAX.

Segundo estudio: con el uso del aCGH se analizaron 19 muestras *BRCA1*, 24 *BRCA2*, 32 BRCAX y 19 muestras de cáncer de mama esporádico. Este estudio fue fruto de una estrecha colaboración con el grupo de Barbara Weber y Katherine L. Nathanson en la Universidad de Pennsylvania de Estados Unidos (que aportó la serie de tumores esporádicos) y el grupo de Michael R. Stratton y Nazneen Rahman de la sección de Genética del Cáncer del Instituto de Investigación del Cáncer (ICR) de Sutton, Inglaterra (que aportó 9 de las 24 muestras *BRCA2*).

Tercer estudio: un subconjunto de los casos del anterior análisis y nuevas muestras hibridadas sobre la misma plataforma de aCGH fueron utilizados en un análisis en el que también se estudió su patrón de IHQ. Los análisis se basaron en un total de 18 *BRCA1*, 16 *BRCA2*, y 28 tumores BRCAX.

Cuarto estudio: centrado en la definición del amplicón 8p11-12 presente en 9 muestras del primer estudio (3 *BRCA1*, 3 *BRCA2* y 3 BRCAX).

Quinto estudio: el amplicón 13q34 ha sido objeto de un último análisis en el desarrollo de esta tesis utilizando 7 muestras (3 *BRCA1*, 1 *BRCA2*, 1 BRCAX, 1 esporádico y 1 línea celular) para su definición genómica y matrices de tejido para la correlación del número de copias y la expresión proteica de uno de los genes candidatos.

1.2. EVALUACIÓN MORFOLÓGICA

En cada uno de estos estudios, los tumores fueron teñidos con hematoxilina-eosina (H-E) y analizados por un patólogo con el fin de establecer el porcentaje de células tumorales, el tipo de tumor y su grado histológico. Solo aquellas muestras que contenían un porcentaje superior a 70% de células tumorales fueron incluidas en los respectivos estudios. Para determinar el grado en los carcinomas ductales infiltrantes, se utilizó el sistema de gradación histológico de Nottingham (Elston and Ellis, 1991).

1.3. LÍNEAS CELULARES

Las líneas celulares utilizadas para el análisis del amplicón 13q34 procedían de distintos laboratorios y fueron cultivadas según las condiciones indicadas a continuación para cada una de ellas e incubadas a 37ºC con 5% CO_2 (Tabla 2). Todos los medios estaban suplementados con fungizona y penicilina/estreptomicina. Antes de proceder a su hibridación en la plataforma de aCGH, se realizaron estudios de hibridación *in situ* con fluorescencia (FISH) para confirmar la existencia del amplicón. Para ello, se realizaron suspensiones citogenéticas de las líneas celulares. Las células de mama crecen adheridas a la superficie de la placa por lo que, tras un lavado con 1xPBS que inhibe cualquier enzima en el medio, se procede a una incubación con 2-4 ml tripsina-EDTA 1x a 37ºC durante 5-

10 min, tras los cuales se recoge con 10 ml. de medio, el cual detiene la reacción de la tripsina. De estos 10 ml., se realizan las diluciones oportunas para mantener el cultivo y, por otra parte, se separan 10 ml de las células en cultivo con el objetivo de detener la mitosis días después añadiendo colchicina (0'1 μg/ml, 1'5 horas, 37ºC; GIBCO, Strachclyde, UK) para obtener un número elevado de células cuyo ciclo celular esté detenido en metafase. Se provoca un choque hipotónico con KCl 75 mM, (30 min., 37ºC) y después de sucesivos lavados se fija con metanol/ acético (3:1) antes de hacer las extensiones sobre los portas.

Tabla 2. Líneas celulares utilizadas.

Línea Celular	Origen	Medio de cultivo
HCC-1937	Dr. P. Edwards, del Dpto. De Patología, Universidad de Cambridge, Cambridge, UK.	90% RPMI + 10% FBS*
MDA-MB-157	Dr. P. Edwards, del Dpto. De Patología, Universidad de Cambridge, Cambridge, UK.	90% DMEM/F12 + 10% FBS*
MDA-MB-436	Dr. K.S. Massey-Brown, del Dpto de Farmacología y Toxicología, Universidad de Arizona, Tucson, USA	90% RPMI + 10% FBS*

* FBS, suero bovino fetal (Gibco-BRL, Grand Island, NY, USA).

Una vez confirmada la amplificación, las hibridaciones en aCGH se realizaron en el laboratorio de Barbara L. Weber y Katherine L. Nathanson de la Universidad de Pennsylvania.

2. EXTRACCIÓN DE ÁCIDOS NUCLEICOS

2.1. EXTRACCIÓN DE ADN DE TEJIDOS CONGELADOS

Como anteriormente se ha mencionado, algunos de los tumores incluidos en los estudios que compusieron esta tesis procedían de tumores frescos preservados con Tissue-Tek® O.C.T. (Sakura Finetek Europe, Zoeterwoude, Netherlands). El protocolo seguido para la obtención de ADN a partir de estas muestras se basa en el protocolo comercial "DNase Tissue Kit" (Qiagen, Chatswoth, CA, USA) con leves modificaciones:

1. Realizar 3-5 cortes de 20µm. de grosor a la muestra tumoral e incluirlos en un tubo eppendorf.
2. Añadir 1 ml. de tampón **PBS** y centrifugar a 12.000 rpm durante 3 min a temperatura ambiente (TA). Desechar sobrenadante. De este modo, se desecha el O.C.T.
3. Repetir paso 2.
4. Resuspender en 180 µl. de tampón **ATL**.
5. Añadir 20 µl. de **proteinasa K** (20 mg/ml), agitar e incubar durante 1-3 h. a 55ºC con agitación de vez en cuando.
6. Añadir 4 µl. de **ARNasa A** (10 mg/ml) durante 5 min. a TA.
7. Añadir 200 µl. de tampón **AL** y mezclar inmediatamente. Incubar durante 10 min. a 70ºC.
8. Añadir 200 µl. de **etanol 100%** y agitar fuertemente.
9. Pipetear la mezcla en las mini-columnas "DNeasy" provistas de tubos colectores de 2 ml.
10. Centrifugar a 8.000 rpm durante 1 min. Desechar sobrenadante.
11. Colocar la mini-columna en un nuevo tubo colector y añadir a la columna 500 µl. de tampón de lavado **AW1**.
12. Repetir paso 10.
13. Añadir a la columna 500 µl. de tampón de lavado **AW2**.
14. Centrifugar a 12.000 rpm durante 3 min.
15. Diluir el ADN retenido en las mini-columnas añadiendo 50 µl. de tampón **AE** a TA. Esperar durante 1 min. a TA.
16. Centrifugar a 8.000 rpm. durante 1 min. No eliminar el volumen diluido.
17. Repetir pasos 15 y 16. Se han recuperado ~100 µl. de volumen en el que está diluido el ADN tumoral.
18. Medir concentración en Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) y correr 500-1.000 µg. en gel de agarosa al 1%.

2.2. EXTRACCIÓN DE ADN DE TEJIDOS PARAFINADOS

2.2.1. Extracción de ADN de tejidos parafinados para estudios de cCGH

De los 80 casos seleccionados para el estudio de cCGH, el ADN se purificó a partir de cuatro secciones de 10 µm de cada tumor siguiendo de nuevo las instrucciones del protocolo comercial "DNase Tissue Kit" (Qiagen, Chatswoth, CA, USA) con algunas modificaciones, el cual se detalla brevemente a continuación:

1. Emplazar en un eppendorf 4 cortes de 10 µm del tumor y añadir 1.200 µl. **xilol** seguido de agitación fuerte para disolver la parafina.
2. Centrifugar a 12.000 rpm. durante 5 min a temperatura ambiente (TA). Desechar sobrenadante.
3. Añadir 1.200 µl. **xilol** seguido de agitación fuerte para disolver la parafina.

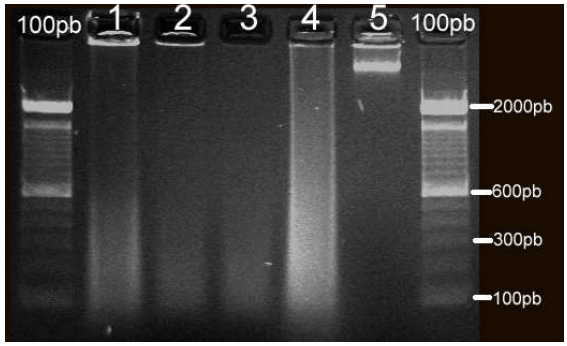
4. Repetir paso 2.
5. Añadir 1.200 µl. de **etanol 100%,** seguido de agitación fuerte.
6. Repetir paso 2.
7. Repetir paso 5.
8. Repetir paso 2.
9. Dejar secar el pellet entre 10-15 min. a 37ºC o a TA.
10. Resuspender en 180/360 µl. de tampón **ATL** dependiendo de la cantidad de tejido.
11. Añadir 20/40 µl. de **proteinasa K** (20 mg/ml) dependiendo de la cantidad de tejido, agitar e incubar durante 1-3 h. a 55ºC con agitación de vez en cuando.
12. Añadir más **proteinasa K** si el tejido no se ha degradado después del tiempo previsto y dejar que la reacción actúe durante toda la noche (16 h.).
13. Añadir 200/400 µl. de tampón **AL** dependiendo de la cantidad de tejido y mezclar inmediatamente. Incubar durante 10 min. a 70ºC.
14. Añadir 200/400 µl. de **etanol 100%** y agitar fuertemente.
15. Pipetear la mezcla en las "DNeasy" mini-columnas provistas de tubos colectores de 2 ml.
16. Centrifugar a 12.000 rpm durante 1 min. Desechar sobrenadante.
17. Repetir pasos 15 y 16 si todavía queda mezcla debido a los volúmenes utilizados previamente dependientes de la cantidad de tejido.
18. Añadir a la columna 500 µl. de tampón **AW1**.
19. Repetir paso 16.
20. Añadir a la columna 500 µl. de tampón **AW2**.
21. Repetir paso 16.
22. Diluir el ADN retenido en las mini-columnas añadiendo 100 µl. de **agua destilada** a 65ºC, o de tampón **Tris-EDTA** (TE 10:1, pH=8.0) o tampón **AE** (provisto por el *kit* comercial) a TA. Esperar durante 5 min. a TA.
23. Centrifugar a 12.000 rpm. Durante 1 min. No eliminar el volumen diluido.
24. Repetir pasos 22 y 23. Se han recuperado ~200 µl. de volumen en el que está diluido el ADN tumoral.
25. Medir concentración en espectrofotómetro y correr 5 µl. en gel de agarosa al 1% para analizar tamaño del ADN. Desechar los ADNs por debajo de 300-500pb.

2.2.2. Extracción de ADN de tejidos parafinados para estudios de aCGH

La extracción de ADN a partir de tejidos parafinados para la hibridación en aCGH fue uno de los grandes problemas a los que tuvimos que hacer frente durante el desarrollo de esta tesis. El ADN debía presentar unos criterios de calidad importantes para ser marcado posteriormente e hibridarlo en los arrays. Hoy en día, existen varios protocolos para medir la calidad del ADN extraído o no de parafina para desecharlo o aceptarlo en estudios de aCGH (Buffart *et al.*, 2007; van Beers *et al.*, 2006). Sin embargo, en el momento en que realizamos las hibridaciones, no existía ninguna prueba de calidad estándar como las mencionadas anteriormente, por lo que la extracción del ADN se cuidó en el más mínimo detalle basándonos en un protocolo de purificación de ADN de tejidos parafinados (http://cc.ucsf.edu/people/waldman/Protocols/paraffin.html) propuesto por De Vries & Waldman, con leves modificaciones. Del protocolo, cabría destacar las incubaciones del tejido en GTE y NaSCN (ver más adelante), encaminadas para eliminar los contaminantes químicos adheridos al ADN por el proceso de fijación en parafina. Posteriormente, se midió la concentración de ADN y se comprobó su tamaño. Toda aquella

muestra cuyo ADN presentaba un tamaño inferior a 300-500 pb. (Figura 7) o su ratio de A260/A280 era inferior a 1'6, no fue utilizada.

Figura 7. Electroforesis en gel de agarosa al 1% de ADNs. Las calles muestran el marcador de peso molecular 100pb de Invitrogen, ADNs de muestras en parafina (1-4) y ADN de una muestra tumoral congelada (5). El ADN de la muestra parafinada 4 tiene un amplio espectro de tamaño y, por lo tanto, es de buena calidad para marcaje, la muestra 1 tiene un tamaño <600 pb (calidad intermedia), mientras que las muestras 2 y 3 tienen un tamaño entre 100-300pb (mala calidad) por lo que no se utilizarán para marcaje. Destaca la diferencia de calidad del ADN de parafina con el ADN de una muestra tumoral congelada.



DÍA 1

1. Cortar 2-3 secciones de 30 µm de los tumores parafinados y meterlos en tubos eppendorf.
2. Añadir 1 ml. de **xilol**, mezclar e incubar durante 15 min. a 55ºC. Liberar presión del tubo y centrifugar durante 2 min. a máxima rpm. para descartar posteriormente el sobrenadante.
3. Repetir el paso 2 una vez más.
4. Añadir 1 ml. de **etanol 100%,** mezclar e incubar durante 15 min. a TA. Centrifugar.
5. Descartar sobrenadante y repetir paso 4.
6. Rehidratar el tejido con lavados sucesivos en 1 ml. de **etanol al 95%, 70%, y 50%**, durante 5 min. en cada uno a TA y seguido de centrifugación a 14.000 rpm. durante 2 min. Descartar en cada lavado el etanol.
7. Añadir 1 ml. de tampón **GTE** (Glycine Tris-EDTA: 100 mM glicina, 10 mM Tris pH 8'0, 1 mM EDTA). Incubar durante 2-5 h. o durante toda la noche a TA.
8. Centrifugar a 14.000 rpm durante 5 min y descartar el GTE.
9. Añadir 1 ml. de 1M **NaSCN** (Sigma, nº catálogo 467871-50G). Incubar toda la noche a 37ºC.

DÍA 2

10. Centrifugar las muestras y desechar el volumen de NaSCN.
11. Añadir 600 µl. de **tampón de digestión** (100mM NaCl/10mM Tris-HCl, pH 8'0 y 25 mM EDTA, pH 8'0/ 0.5%SDS) y 30 µl. de **proteinasa K** (20 mg/ml) para una concentración final de ~1 mg/ml. Reducir o aumentar cantidades dependiendo del tamaño del tejido.
12. Incubar durante 2-3 h. o toda la noche y en movimiento a 55ºC.
13. Añadir más proteinasa K si el tejido no está disuelto y repetir paso 12.

DÍA 3

14. Centrifugar unas columnas "Phase-Lock Gel" (PLG) a 12.00-16.000 rpm. durante 30 seg.
15. Pasar la fase acuosa de la digestión con proteinasa K a los tubos centrifugados PLG.
16. Añadir un volumen igual de **fenol cloroformo isoamílico** (FCI, 25:24:1, Sigma, nº catálogo P3803).
17. Mezclar la fase acuosa y orgánica invirtiendo el tubo repetidas veces. Incubar durante 5 min.
18. Centrifugar a 12.000-16.000 rpm durante 5 min. para separar las fases. Extraer fase acuosa.
19. Repetir pasos 16-18 con otros tubos si es necesario por haber demasiado volumen o para purificar la fase acuosa (en la que está diluido el ADN).
20. Cuando no se necesiten hacer más extracciones FCI, mezclar la fase acuosa con 1 volumen de **cloroformo isoamílico** (CI, 24:1, Sigma, nº catálogo C0549-1PT). Repetir pasos 17 y 18.
21. Tomar 330 µl. de la fase acuosa por tubo eppendorf para precipitar el ADN. Añadir ½ de volumen (165 µl.) de **acetato amónico** (7'5M) ó 1/10 volumen (33 µl.) de **NaOAC** (pH 7'5). Añadir 1 µl. de glucógeno (10 µg/µl) para preservar las pequeñas cantidades de ADN y hacer que el precipitado sea visible. Añadir 2 volúmenes (660 µl.) de **etanol 100% frío**. Mantener a -20ºC durante 2 h. o toda la noche.

DÍA 4

22. Centrifugar durante 20-30 min. a 4ºC, decantar etanol, lavar el precipitado con 500 µl. de **etanol 70%** y centrifugar 10 min. a 4ºC.
23. Decantar todo el etanol que sea posible sin tocar el precipitado y dejar secar al aire.
24. Resuspender el precipitado con cuidado en agua destilada o en **Tris-EDTA** (TE 10:1, pH=8'0) y dejar disolver a TA durante toda la noche o a 55ºC durante 2 h.
25. Medir concentración de ADN en espectrofotómetro y correr 0'5-1 µg. de ADN en un gel de agarosa al 1% para comprobar tamaño y calidad. El tamaño debe ser superior a 300 pb.

3. HIBRIDACIÓN IN SITU CON FLUORESCENCIA (FISH)

La hibridación *in situ* con fluorescencia permite la localización de secuencias de ADN específicas sobre preparaciones cromosómicas, extensiones celulares y tejidos congelados o parafinados. Esta técnica se basa en la hibridación de un fragmento de ADN marcado con un fluorocromo (sonda) sobre su secuencia complementaria del genoma de la muestra. Existen tres tipos de sondas:

- Sondas centroméricas: son aquellas que hibridan con las regiones centroméricas de los cromosomas. Estas sondas permiten detectar alteraciones numéricas (ganancias o pérdidas de cromosomas enteros).
- Sondas de pintado cromosómico: hibridan a lo largo de todo un cromosoma y permiten detectar alteraciones numéricas y estructurales sobre metafases.
- Sondas de secuencia única: estas sondas hibridan con una región específica del cromosoma, que puede ser un gen u otra región.

Las sondas empleadas pueden ser comerciales (para la detección de alteraciones típicas en el diagnóstico) u obtenidas a partir de cromosomas artificiales (generalmente BACs), marcados con fluorescencia mediante el método del desplazamiento de la mella.

3.1. OBTENCIÓN DE SONDAS PARA FISH

3.1.1. Diseño de las sondas

A partir de la secuenciación del genoma humano se ha generado una gran cantidad de información sobre librerías de clones, tanto localizados como secuenciados que son de gran utilidad en la citogenética molecular. Actualmente se puede obtener información de las regiones cromosómicas en distintas bases de datos: *Ensembl* (http://www.ensembl.org), *National Center for Biotechnology Information* (http://www.ncbi.nlm.nih.gov) y el buscador genómico de la Universidad de Santa Cruz de California (UCSC) (http://www.genome.cse.ucsc.edu/index.html). Estos buscadores permiten la localización de clones que cubran las regiones de interés, ya que muestran la posición relativa de los genes y los distintos BACs (cromosomas artificiales derivados de bacterias) y PACs (cromosomas artificiales derivados de P1). Los BACs son los más utilizados. Estos clones permiten el aislamiento de fragmentos de ADN plasmídico de entre 100-200 Kb que son lo

suficientemente grandes como para utilizarlos en estudios de FISH. Estos cromosomas artificiales se pueden adquirir fácilmente en *Children Hospital Oakland Research Institute* (http://www.chori.org/bacpac/) o en *CalTech BAC* (http://www.informa.bio.caltech.edu/).

3.1.2. Cultivo de los clones bacterianos

Los clones se suministran en LB sólido e inestable que limita su viabilidad. A partir de estos clones bacterianos se realiza un cultivo en medio sólido para el aislamiento de colonias, mediante una siembra por agotamiento de asa, a 37ºC toda la noche. El medio utilizado es LB (Luria Bertani, 10 g/l *Bacto-tryptone*, 7'5 g/l *Bacto Yeast Extract* y 10 g/l NaCl a pH 7'5) suplementado con cloranfenicol 20 µg/ml o kanamicina 25 µg/ml dependiendo del tipo de clon utilizado. Para obtener una cantidad suficiente de ADN se seleccionó una de las colonias y se cultivó en 10 ml de LB líquido suplementado con antibiótico, durante toda la noche, a 37ºC con agitación (250 rpm).

Para preservar los clones se congelan dos viales de cada clon con 800 µl. del cultivo líquido, con 200 µl. de glicerol estéril. Los sucesivos cultivos se realizan a partir de estos reservorios.

3.1.3. Extracción del ADN plasmídico

La extracción de ADN plasmídico se realizó con el método de lisis alcalina. Brevemente, las bacterias se someten a una lisis de la membrana bacteriana. Después se solubilizan las proteínas con un detergente y las membranas se precipitan con isopropanol. Finalmente los plásmidos son precipitados con acetato sódico y etanol absoluto. El protocolo utilizado fue el siguiente:

1. Centrifugar el medio con las bacterias durante 10 min. a 4000 rpm. Eliminar el medio de cultivo y nos quedamos con el precipitado (*pellet*) bacteriano.
2. Resuspender el *pellet* con 300 µl de **GTE** (Glucosa 50 mM, Tris 25 mM pH=8, EDTA 10 mM). Transferir la suspensión celular a un tubo limpio y poco a poco añadir 600 µl de **solución alcalina** (0'2 N NaOH, 1% SDS) recién hecha. Mezclar por inversión varias veces e incubar 5 min. en hielo.
3. Añadir 500 µl de **acetato amónico** 7'5 M para separar las proteínas y el ADN bacteriano de los plásmidos. Mezclar inmediatamente invirtiendo varias veces y dejar en hielo 10 min.
4. Centrifugar a 13.000 rpm durante 10 min. a 4ºC. Pasar el sobrenadante a un tubo limpio y repetir este paso.
5. Precipitar el ADN plasmídico añadiendo 700 µl de **isopropanol** y centrifugando a 14.000 rpm. durante 20 min. a 4ºC. Descartar sobrenadante y lavar *pellet* con 500 µl **etanol 70%**.

6. Resuspender el *pellet* de ADN en 100 µl de **agua** y tratar con 1 µl de **ARNasa** (10 mg/ml) durante media hora a 37ºC para eliminar el ARN bacteriano que ha precipitado.
7. Volver a precipitar los BACs con 1/10 volúmenes **acetato sódico** (3 M y pH 5'5) y 2'5 volúmenes de **etanol absoluto**. Incubar a –80ºC durante 20 min. Centrifugar 15 min. a 14.000 rpm. y lavar con **etanol 70%**.
8. Resuspender en **agua estéril** (100 µl). Medir la concentración en NanoDrop® ND-1000 (Innovadyne Technologies, Inc, Santa Rosa, California) y comprobar la calidad cargando 5 µl en un gel de agarosa (1% m/v).

3.1.4. Marcaje de la sonda

Una vez extraídos los ADN de interés, se procede al marcaje mediante el método de desplazamiento de la mella ("nick translation"). El marcaje consiste en la incorporación de dUTPs conjugados con fluorocromos en el ADN. Este marcaje es posible gracias a la acción de dos enzimas, ADNasa I y ADN polimerasa I de *Escherichia coli.* La ADNasa I tiene actividad endonucleasa, es capaz de producir mellas al azar en el ADN. Estas roturas permiten la adición de nuevos nucleótidos, que pueden estar conjugados con fluorocromos, mediado por la actividad polimerasa 5´→3 de la ADN polimerasa I. Al mismo tiempo se eliminan los nucleótidos adyacentes mediante la actividad 5´→3´ exonucleasa de la polimerasa. De este modo la mella se va desplazando en sentido 5´→3´ (Figura 8). Cuando se encuentran cortes en las dos hebras, el ADN se fragmenta. Como los fragmentos se producen al azar, el ADN se marca uniformemente.

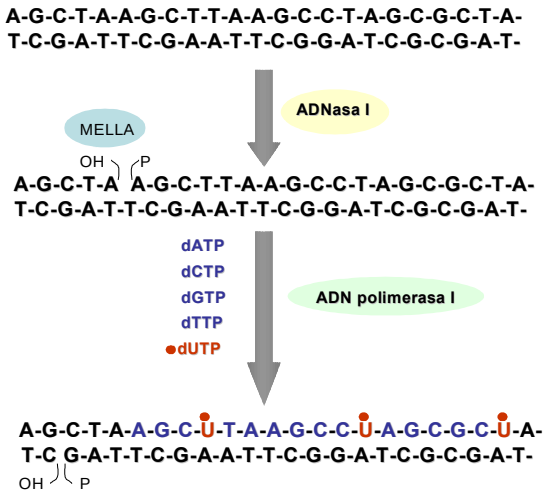


Figura 8. Marcaje de ADN mediante desplazamiento de la mella. La ADNasa I introduce mellas al eliminar el enlace fosfodiéster entre las bases; se genera un extremo 3'OH y un 5' con un fosfato libre. La ADN polimerasa incorpora los nuevos nucléotidos en sentido 5'→ 3' mientras va desplazando la mella eliminando nucleótidos a partir del extremo 5' libre.

El marcaje se realiza con "CGH Nick Translation Kit" (Vysis, Downers Grove, USA). Este kit incluye las enzimas ADNasa I y ADN polimerasa I, el tampón de reacción 10X y los dNTPs sin conjugar. Brevemente el protocolo es el siguiente:

1. Preparar la mezcla de **dNTPs** 0'1 mM.: 10 µl de dATP 0'3 mM., 10 µl dGTP 0'3 mM. y 10 µl dCTP 0'3 mM.

2. Preparar la solución de **dTTP** 0'1 mM. diluyendo tres veces los dTTPs 0'3 mM. con agua.
3. Preparar el **SpectrumGreen** (SG) o **SpectrumRed** (SR) dUTP (Vysis, Downers Grove, USA) 0,2 mM. El **dUTP** viene liofilizado, se resuspende con 50 µl de agua estéril y después, se diluyen 10 µl de dUTP (SG o SR) con 40 µl de agua estéril.
4. En un tubo se mezclan:

ADN	**X**µl
Tampón 10x	5µl
dNTPs 0.1mM	5µl
dTTP 0.1mM	5µl
SR/ SG dUTP 0.2mM	2µl
Enzimas	10µl
H_2O.	**Y**µl
Volumen final	50µl

5. La mezcla se incuba durante 2 horas a 15ºC. Después se carga un gel de agarosa 1% (m/v) para comprobar que los fragmentos se encuentran 250 pb-1000 pb, si está muy por encima volver a incubar un poco más.
6. Cuando los fragmentos se encuentran en el tamaño adecuado, la mezcla se incuba a 70ºC durante 10 min. para que se produzca la ruptura de la enzima.
7. Después, purificar el ADN con *QIAquick PCR Purification Kit* (Qiagen GmbH, Hilden, Alemania) siguiendo las recomendaciones de la casa comercial. Añadir 5 volúmenes de **tampón PB**, poner la mezcla en la columna y centrifugar a 13.000 rpm durante 1 min. En esta centrifugación se eliminan los nucleótidos no incorporados que podrían interferir en la hibridación posterior. Lavar con 750 µl de **tampón PE** y centrifugar 1 min. A continuación, el ADN marcado se eluye con 100 µl de **agua estéril** a 65ºC durante 5 min.
8. Finalmente, precipitar la sonda junto con 20 µl de **Human Cot-1® DNA** (1 µg/µl) (Roche Diagnostics GmbH, Mannheim, Alemania), con 1/10 vol. de **acetato sódico** (3 M, pH 5) y 2'5 vols. de **etanol absoluto**. Centrifugar y eliminar el sobrenadante y se resuspende en 10 µl. de **agua estéril**. El Cot 1 son secuencias de ADN que bloquean secuencias repetitivas.

3.2. PROTOCOLO DE FISH

3.2.1. Protocolo de FISH en muestras parafinadas

La mayor parte de los análisis FISH realizados tuvieron como objeto de estudio muestras parafinadas, las cuales fueron incluidas en matrices de tejidos (*tissue microarrays* o TMA, ver epígrafe 6.2 de Material y Métodos). Se utilizaron secciones de 4 µm. de los TMA y se siguió el siguiente protocolo:

TRATAMIENTO DE LA MUESTRA EN PARAFINA – DÍA 1

1. Colocar los portas durante toda la noche en estufa a 65ºC.
2. Desparafinar los portas en sucesivos lavados: 2x en **xilol** a 65ºC durante 10 min., 2x en **etanol absoluto** a TA durante 5 min., 1x en **etanol 80%** 5 min., 1x en **etanol 70%** 5 min. y emplazar en **agua destilada** hasta siguiente paso.
3. Llenar una olla con ½ litro (lo suficiente como para cubrir los portas) de **EDTA** 1 mM. pH 8.0 (2 ml. EDTA líquido en 1 l. de agua y ajustar pH). Calentar en microondas.
4. Emplazar los portas en una rejilla dentro de la olla, cerrarla y hervir durante 4-8 min. en funcion del tipo y tamaño de tejido.

5. Pasar los portas a **1xPBS ó agua destilada**.
6. Tratamiento con pepsina: colocar 40 µl. de la solución con **pepsina** (10 ml. H2O, 100 µl. HCl 1M y 5 µl. de pepsina) sobre el corte, cubrirlo con *parafilm* y emplazar el cristal en una cámara húmeda a 37ºC durante 30 min. Después se introducen en una **solución de parada** (50 ml. PBS, 2.5 ml. Mg_2Cl 1M) durante 5 min.
7. Lavar en agua y dejar secar los portas.

FISH DE LA MUESTRA EN PARAFINA – DÍA 1

8. Preparación de la sonda: 1 µl. de la sonda se diluye con 2 µl de **agua** y 7 µl. de **tampón de hibridación LSI/WCP** (Vysis, Downers Grove, USA), que contiene sulfato de dextrano, formamida y SSC pH 7. Meter la sonda a 37ºC durante 30-45 min.
9. Preparación de la muestra: deshidratar en etanol 70, 80 y 100% durante 4 min. cada uno, y 5 min. en estufa a 65ºC para eliminar todo el alcohol.
10. Incubar la sonda a 96ºC durante 5 min.
11. Poner la sonda y tapar con el cubre.
12. Desnaturalizar durante 1'30-2 min. a 80ºC.
13. Envolver el porta con *parafilm* y emplazarlo en una cámara húmeda a 37ºC durante toda la noche.

LAVADO DE LA FISH DE LA MUESTRA EN PARAFINA – DÍA 2

14. Primer lavado en jarra *coplin* con **tampón 1** (0'4xSSC, 0'3%NP40) durante 2 min. a 75ºC.
15. Segundo lavado con **tampón 2** (2xSSC, 0'1%NP40) durante 5 min. en agitador y a TA.
16. Lavar en **1xPBD** durante 2 min. a TA.
17. Contrateñir con una mezcla 1:1 de **DAPI** (Vysis, Downers Grove, IL, USA) y **solución antidecaimiento o antifade** (Qbiogene, Heidelberg, Alemania). El DAPI (4'-6'diamidino-2-fenilindol) se intercala en el ADN y permite su visualización de color azul cuando se excita con una longitud de onda determinada.

3.2.2. Protocolo de FISH en suspensiones citogenéticas

En el desarrollo de esta tesis, también se realizaron estudios de FISH en suspensiones citogenéticas procedentes de cultivos de líneas celulares. El protocolo es similar al de la FISH en muestras de parafina salvo en algunos pasos: en la preparación de la muestra, antes de la deshidratación con etanoles, se envejece la muestra a 90ºC durante 10 min; y la desnaturalización de la muestra y la sonda es a 75ºC durante 1 min.

3.3. CAPTURA Y ANÁLISIS DE LAS IMÁGENES

Para la captura de imágenes se utilizó un microscopio epifluorescente (Olympus AX60) acoplado a una cámara digital (Sensys). La captura y posterior análisis de las imágenes se realizó con el programa "CytoVision Image Analysis System" (Applied Imaging, Newcastle, UK). El recuento del número de señales de fluorescencia fue realizado en cada muestra contando el número de señales del gen y el número de señales centroméricas o de control en un promedio de 130 (60-210 núcleos).

3.4. DESCRIPCIÓN DE LAS SONDAS UTILIZADAS

A lo largo de esta tesis, se han utilizado diferentes sondas FISH para el estudio de número de copias (amplificación y deleción) de diversas regiones cromosómicas o genes de interés. A continuación se detallan las sondas utilizadas, su localización cromosómica, el marcaje usado y el criterio para definir el resultado.

3.4.1. Análisis de dosis génica de dos regiones cromosómicas

Como parte del segundo estudio de la presente tesis y durante el transcurso del análisis de los resultados, se procedió a una estandarización de un patrón de artefactos descrito paralelamente en los aCGH de muestras parafinadas (ver Material y Métodos 5.1.3). Para validar la estandarización, se comparó los valores del número de copias del aCGH estandarizado con los valores obtenidos en un estudio FISH realizado sobre matrices de tejidos que contenían las muestras parafinadas. Se comprobó el número de copias de dos regiones cromosómicas diferentes afectadas por los artefactos: 1p36 y 22q11-q12 (Figura 11C-D). La sonda 1p36 contenía tres clones BAC de la región distal del brazo corto del cromosoma 1 (RP11-82D16, RP4-713A8 y RP4-740C4, localizados a 2'07 Mb, 2'25 Mb y 2'30 Mb del telómero de 1p respectivamente). Todos estos BACs se marcaron con dUTP-SpectrumGreen (Vysis, Inc. Downer's Grove, IL, USA). La sonda 1p36 también contenía una sonda centromérica del cromosoma 1 "CEP1 alpha satellite DNA Spectrum Orange" de Vysis, Inc. (Downer's Grove, IL, USA). Por su parte, la sonda 22q11-q12 contenía dos clones localizados en 22q11.21 (RP11-316L10 y RP11-330P17) marcados con dUTP-SpectrumGreen, y tres clones localizados en 22q12.2 (RP1-76B20, RP1-15I23 y RP3-394A18) marcados con dUTP-SpectrumOrange. Se contaron el número de copias de cada región y de sus señales centroméricas.

3.4.2. Análisis de la amplificación de genes de interés

Se realizó el análisis FISH de tres genes importantes en el desarrollo del cáncer de mama: *ERBB2*, *c-MYC* y *CCND1* con el objeto de establecer asociaciones siempre que fuera posible.

Para la detección de la amplificación de *ERBB2*, se utilizó la sonda comercial de Vysis (Downer's Grove, IL, USA). La sonda mide 120 Kb, cubre el gen *ERBB2* por completo y está marcada con SpectrumOrange. También se incluye una sonda que hibrida

en el ADN alfa satélite del centrómero del cromosoma 17 (17p11.1-q11.1) y que está marcada en SpectrumGreen.

Para la amplificación de *c-MYC*, utilizamos la sonda tricolor *IGH/MYC/*CEP8 de Vysis (Downer's Grove, IL, USA). Esta sonda es una mezcla de una sonda de IGH de 1'5 Mb marcada con SpectrumGreen, de una sonda de *c-MYC* de 750 Kb. marcada con SpectrumOrange y de la sonda del centrómero del cromosoma 8 (CEP8) marcada con SpectrumAqua. La sonda *c-MYC* cubre el gen por completo, mientras que la sonda CEP8 cubre el ADN alfa satélite del cromosoma 8.

Para la amplificación de *CCND1*, se usó la sonda comercial de Vysis (Downer's Grove, IL, USA) que cubre el gen al completo y está marcada con SpectrumOrange. Se usó también una sonda centromérica del cromosoma 11, marcada con SpectrumGreen y que hibrida en el ADN alfa satélite del cromosoma 11 (11p11.1-q11.1).

La amplificación fue definida como la presencia (en >5 % de células del tumor) de >10 señales del gen para *ERBB2* y >6 para C-*MYC* y *CCND1*.

3.4.3. Análisis de un nuevo amplicón (13q34)

En el estudio de la amplificación de una región poco estudiada como 13q34, se realizaron análisis FISH para validar los niveles de amplificación obtenidos por aCGH o para buscar más casos amplificados con el objetivo de incluirlos en el estudio. La sonda contenía tres clones BAC de la región 13q12.11 marcados con dUTP-SpectrumGreen (Vysis Inc., Downer's Grove, IL, USA): RP11-301J16 (18'52 Mb), RP11-408E5 (18'67 Mb) y RP11-385E5 (18'81 Mb). La sonda también incluía tres clones BAC localizados en la región 13q34 y marcados con dUTP-SpectrumOrange (Vysis Inc., Downer's Grove, IL, USA): RP11-391H12 (113 Mb), RP11-102K13 (113'17 Mb) y RP11-230F18 (113'26 Mb). La amp13q34 se definió como la presencia (en >5% de células del tumor) de tres veces tantas señales de la región control 13q12.11.

4. HIBRIDACIÓN GENÓMICA COMPARATIVA SOBRE CROMOSOMAS O CONVENCIONAL (cCGH)

La CGH (por las siglas en inglés "Comparative Genomic Hibridization"), descrita a principio de los noventa (Kallioniemi et al., 1992), se basa en la hibridación competitiva de dos ADNs (tumoral y control normal) marcados con distintos fluorocromos, sobre cromosomas normales que permite la detección de ganancias y pérdidas en todo el genoma. La resolución media es de 10 Mb (Kallioniemi *et al.*, 1994b) aunque con los nuevos programas de alta resolución podría llegarse hasta una resolución de 3Mb (Kirchhoff *et al.*, 1999b). Esta técnica emplea ADN del tumor, obviando la necesidad de células en crecimiento. En resumen: se marca el ADN del tumor con un fluorocromo verde y un ADN normal (control) con un fluorocromo rojo. Ambos ADNs se mezclan en cantidades equimolares y se realiza una hibridación *in situ* sobre cromosomas metafásicos normales. Ambos ADNs compiten por hibridar en los mismos lugares cromosómicos. En condiciones normales (tumor sin alteraciones genéticas), como la cantidad de ADN marcado en rojo y verde es la misma, el resultado final son cromosomas amarillos. En condiciones patológicas (si el tumor contiene alguna ganancia cromosómica), la cantidad de ADN tumoral disponible para hibridar es mayor, y la hibridación de esa zona resultará en una mayor proporción de fluorocromo del tumor (verde). Al contrario, si el tumor contiene una deleción (pérdida), la región perdida del tumor aparecerá en rojo, ya que habrá más cantidad de ADN normal (rojo) para hibridar en esa región cromosómica (Figura 9).

4.1. MARCAJE DE LOS ADN MEDIANTE DESPLAZAMIENTO DE MELLA

El marcaje se realiza mediante el método de desplazamiento de mella (ver apartado MM 3.1.4). Brevemente, se marcó alrededor de 1'5 µg ADN genómico de las muestras de parafina de cáncer de mama con SpectrumGreen-dUTP mediante el "CGH Nick Translation Kit" (Vysis Inc., Downer's Grove, IL, USA). Al tratarse de ADN de tejidos parafinados, el período de marcaje se reduce a 45 min.-1h 15min. para evitar una mayor degradación del ADN. Los fragmentos óptimos para cCGH deben estar entre 250-2.000 pb (Figura 10).

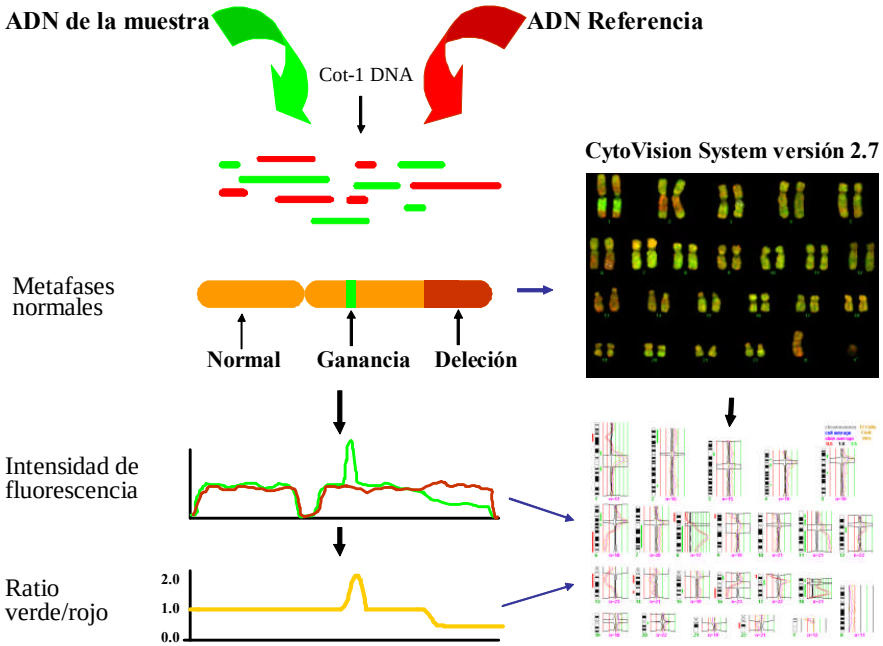


Figura 9. Esquema de cCGH. Las muestras se marcan diferencialmente, en rojo el control y en verde la muestra, y se co-hibridan sobre cromosomas metafásicos. Una vez colocados se calculan la intensidad de fluorescencia de cada canal a lo largo del cromosoma y después se calcula el ratio verde/rojo, que nos proporciona los perfiles de ganancia y pérdida.

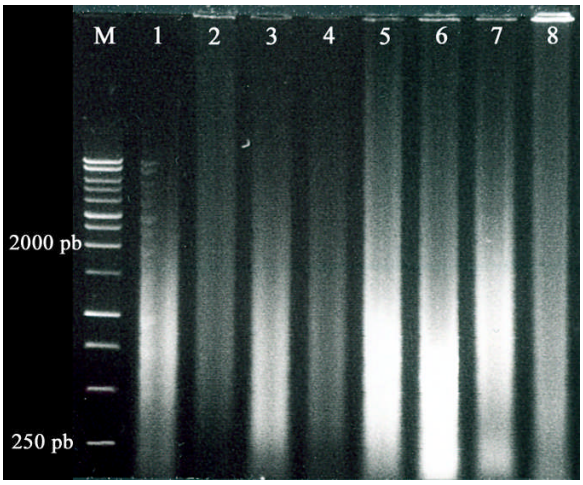


Figura 10. ADNs marcados mediante desplazamiento de la mella. M: marcador de peso molecular 1Kb (Biotools, Madrid, España). Calles de 1 a 8: muestras de parafina que tienen un buen rango de tamaños de ADN marcado, salvo en el caso de las muestras 2 y 4 que todavía tienen unos tamaños altos de ADN y debería marcarse unos minutos más.

4.2. PLATAFORMA DE cCGH: LAS METAFASES NORMALES

Como diana de nuestra sonda se utilizan portaobjetos con suspensiones citogenéticas de individuos sanos. La calidad de las metafases debe ser óptima. No deben presentar citoplasma, los cromosomas deben estar expandidos, tener una longitud que alcance la resolución de 400-500 bandas y no deben ser refringentes al observarlos en un microscopio de contraste, esto indica que pueden desnaturalizarse con facilidad (Karhu *et al.*, 1997). Por todo ello, se utilizaron preparaciones citogenéticas con metafases normales de varón comerciales (Vysis, Downers Grove, USA).

4.3. PROTOCOLO DE cCGH

MARCAJE DEL ADN DE LA MUESTRA – DÍA 1

1. Marcaje del ADN de la muestra mediante el método de desplazamiento de mella (ver apartados MM 3.1.4. hasta el paso 7 incluído y MM 4.1).

PREPARACIÓN DE LA SONDA – DÍA 1

2. Precipitación del ADN con 30 µl. de **Human Cot-1® DNA** (1 µg/µl) (Roche Diagnostics GmbH, Mannheim, Alemania), 2 µl. de **ADN referencia**, 1/10 vol. de **acetato sódico** (3 M, pH 5) y 2'5 vols. de **etanol absoluto**. Centrifugar y eliminar el sobrenadante y se resuspende en 10 µl. de **agua estéril**. El Cot 1 son secuencias de ADN que bloquean secuencias repetitivas. Como ADN de referencia, se utilizó un ADN comercial de un varón normal marcado con Texas Red dUTP (Vysis Inc, Downer's Grove, IL, USA). Almacenar a -80ºC durante 1 h. o a -20ºC durante toda la noche. Centrifugar a velocidad máxima a 4ºC durante 30 min.
3. Retirar sobrenadante y dejar secar la mezcla de ADN en oscuridad.
4. Añadir 4'5 µl. de **agua** y 10,5 µl. de **tampón de hibridación LSI/WCP** (Vysis, Downers Grove, USA). Resuspender bien.
5. Desnaturalización de la sonda a 75ºC durante 10 min.
6. Incubar a 37ºC durante 30 min. para el pre-anillamiento (paso opcional).

PREPARACIÓN DE LAS METAFASES NORMALES – DÍA 1

7. Deshidratar las preparaciones metiéndolas en una serie de alcoholes de gradación creciente (70%, 80% y 100%) durante 3 min. en cada uno.
8. Dejar secar y pasar a una solución de **formamida** (35 ml formamida desionizada, 5 ml 20xSSC y 10 ml de agua) a 75ºC durante 1 min. para desnaturalizar los cromosomas.
9. Deshidratar inmediatamente se deshidratan con una nueva serie de alcoholes fríos (-20ºC) y dejar secar a TA.

HIBRIDACIÓN – DÍA 1

10. Añadir la sonda sobre los portas. Cubrir con cubres de 24x24 y envolver en *parafilm*.
11. Incubar en cámara húmeda en estufa a 37ºC durante 48-72 horas.

LAVADOS POST-HIBRIDACIÓN Y TINCIÓN DE CONTRASTE - DÍA 2

12. Como en el protocolo de FISH (ver MM 3.1.4.), los cristales se pasan por una solución 0'4xSSC con 0'3% de NP-40 a 75ºC durante dos minutos. Después, poner en una solución de 2xSSC con 0'1% de NP.40 a TA, 5 min. en agitación. Finalmente, se lavan en PBD1X a T.A. dos minutos más. Se deja escurrir el exceso de PBD1X.
13. Teñir los cromosomas con 20 µl de 1:1 DAPI y *antifade*.

4.4. CAPTURA Y ANÁLISIS DE LAS IMÁGENES

Una vez realizados los lavados, se procedió a la captura de 10 a 15 metafases de cada caso con la cámara digital CCD (Photometrics, Inc., Tucson, AZ, USA) acoplada a un microscopio Olympus AX60 de epi-fluorescencia. Los ratios de señal verde/rojo se calcularon utilizando el software "CytoVision system versión 2.7" (Applied Imaging, Newcastle, United Kingdom) para el análisis de CGH de alta resolución (HR-CGH). Este método de análisis de CGH se basa en los intervalos de referencia estándar dinámicos para cada muestra. Este método permite una mayor resolución en la detección de alteraciones, hasta 3Mb, y permite el estudio de las regiones excluidas en los sistemas tradicionales, 1p, 19, 22 y las regiones teloméricas (Kirchhoff *et al.*, 1999a; Kirchhoff *et al.*, 1998; Kirchhoff

et al., 2001). El cálculo de los ratios de las intensidades se realiza una vez que se hayan ordenado los cromosomas adecuadamente. En general, un cociente cercano a 1, indica que la cantidad de ADN en esa región es la misma en la muestras como en el control; cuando el ratio es superior a 1'25 hay mayor cantidad de ADN de la muestra, por lo que la intensidad del verde es mayor y eso representa una ganancia de la región; por el contrario cuando el ratio es menor de 0'75 hay una menor cantidad de ADN de la muestra, por lo que la intensidad será mayor en el canal rojo, por lo que habrá una perdida en esa región. Sin embargo, cuando utilizamos la CGH de alta resolución se compara la media de los perfiles de los ratios de cada caso, con un intervalo de confianza del 95%, con la media de los perfiles de controles normales con el mismo intervalo de confianza. Las regiones alteradas son aquellas en que los intervalos de confianza de la muestra y el control no se superponen.

Se utilizaron hibridaciones de ADN de muestras normales de tejido de mama en parafina frente a ADN de referencia como controles negativos antes del análisis de las muestras de tumores. Dado que las hibridaciones se realizaron enfrentando ADN de sexos opuestos, los cromosomas sexuales (X e Y) fueron omitidos de posteriores análisis.

Para definir el patrón de HR-CGH de cada caso, se definieron 63 regiones citogenéticas de bandas G tal y como se describió previamente en un trabajo de nuestro grupo (Alvarez *et al.*, 2005). Brevemente, se seleccionaron las 50 regiones mínimas de alteración más comunes, que presentaban aberraciones en al menos 30% de los casos *BRCA1/2* utilizados para la construcción del clasificador y con al menos tres de ellos definiendo los límites citogenéticas de la región. Para incluir el resto del genoma que no quedaba englobado en estos criterios, se definieron otras 13 regiones cromosómicas. Posteriormente, se dio un valor discreto por cada región en función de la aberración que presentaba: no cambio (0), ganancia (1) o deleción (-1).

5. HIBRIDACIÓN GENÓMICA COMPARATIVA SOBRE MATRICES DE CLONES (ARRAY DE CGH O aCGH)

Para el desarrollo de esta tesis, se emplearon dos plataformas diferentes de array de CGH: una que cubría por completo el genoma humano a una resolución de 1 Mb. (utilizado para los estudios 2, 3 y 5) y otra plataforma específica del cromosoma 8 (para la elaboración de nuestro cuarto estudio).

5.1. PLATAFORMA DE ARRAY DE CGH GLOBAL DEL GENOMA

La hibridación genómica comparativa de las muestras embebidas en parafina de cáncer de mama familiar y de las muestras congeladas de cáncer de mama esporádico se realizó sobre una plataforma de aCGH desarrollada en la Universidad de Pennsylvania (Greshock *et al.*, 2004), previamente utilizada en estudios similares (Jonsson *et al.*, 2005; Naylor *et al.*, 2005). La plataforma está compuesta de 4.134 clones BAC espaciados con una resolución de 1 Mb. cubriendo todo el genoma. El ADN de los BACs fue amplificado mediante reacciones en cadena de la polimerasa con cebadores de oligonucleótidos degenerados (DOP-PCR). Se imprimieron al menos dos réplicas de cada clon BAC en cada cristal usando el "Lucidea Array Spotter" (Amersham Biosciences) y una solución de 50% DMSO (Greshock *et al.*, 2004).

5.1.1. Protocolo de hibridación de aCGH sobre la plataforma de 1 Mb

Por cada muestra, se realizaron dos hibridaciones utilizando fluorocromos opuestos (hibridación "dye-swap") para contabilizar las diferencias en la incorporación del marcaje y ofrecer una mayor cantidad de puntos para el análisis.

MARCAJE DEL ADN MEDIANTE CEBADO ALEATORIO – Bioprime Labeling System (Invitrogen) - DÍA 1

1. 1'5 µg. de **ADN de la muestra** y 1'5 µg. del **ADN referencia** se marcarán por separado usando el kit *Bioprime® Labeling System* (Invitrogen). El ADN referencia utilizado procede de un pool de ADN de mujeres de diferentes etnias.
2. Llevar a 22 µl. con **agua** y añadir 20 µl. de **cebadores aleatorios** (*random primers*).
3. Desnaturalizar durante 15 min. a 95ºC.
4. Guardar en hielo durante 15 min.
5. Crear la mezcla de reacción añadiendo: 5'0 µl. de la mezcla de **dNTP** (12 µl. de dATP 100mM + 12 µl. de dGTP 100mM + 12 µl. de dTTP 100mM + 6 µl. de dCTP 100mM + 958 µl. de Tris-EDTA pH 8'0) + 2'0 µl. de fluorocromos **Cy3-dCTP** ó **Cy5-dCTP** (Amersham Biosciences) + 1'0 µl. de **Fragmento Klenow**.
6. Incubar en oscuridad a 37ºC durante toda la noche.

PURIFICACIÓN – MinElute PCR purification Kit (Qiagen) – DÍA 2

7. Añadir 5 volúmenes de **tampón PB** a 1 volumen de reacción (250 µl. PB a 50 µl. de reacción).
8. Emplazar el volumen total en una columna MinElute.
9. Centrifugar a máx. rpm. durante 1 min.
10. Descartar el eluido y añadir 750 µl. de **tampón PE**.
11. Repetir paso 9.
12. Descartar el eluido. Centrifugar a máx. rpm durante 1 min. para eliminar restos de PE.
13. Poner la columna en un tubo nuevo de 1'5 ml. convenientemente etiquetado.
14. Eluir el ADN, añadiendo 12 µl. de tampón EB (10mM Tris-Cl, pH 8'5) o agua (a pH 8'0) en el centro de la columna, dejar durante 5 min. a TA y a oscuras. Centrifugar 1 min.

MEZCLA DE SONDAS, PRECIPITACIÓN DEL ADN Y LAVADO – DÍA 2

15. Combinar los ADN marcados de referencia y de la muestra obtenidos del paso 14.
16. Añadir 100 µl. de **Human Cot-1® DNA** (1 µg/µl) (Roche Diagnostics GmbH, Mannheim, Alemania) + 20 µl. de **ARNt** + 1/10 vol. de 3M **acetato sódico** (pH 7'0 ó 5'2) ó de **acetato amónico** 5M (recomendable para ADNs marcados) + 2'5 vols. de **etanol 100%** frío.
17. Precipitar a -20ºC durante 1-2 h. o durante toda la noche.
18. Centrifugación a máxima velocidad y a -4ºC durante 20-30 min.
19. Decantar sobrenadante.
20. Lavar el precipitado (de color púrpura) con 500 µl. de **etanol 70%** frío.
21. Centriguración durante 10 min.
22. Decantar sobrenadante.
23. Dejar secar la sonda en oscuridad durante 15 min. a TA.
24. Congelar la muestra a -20ºC o rehidratar con 50 µl. de tampón de hibridación (para 5 ml. de tampón de hibridación que posteriormente se repartirá en tubos de 1'5: 2'5 ml. de formamida desionizada, 500 mg. de dextrán-sulfato, 500 µl. de 20xSSC, 1 ml. de 10% SDS, 800 µl. de agua destilada).

PREPARACIÓN DEL CRISTAL PARA LA HIBRIDACIÓN – DÍA 2

25. Precalentar a 42ºC el **tampón de pre-hibridación** (para 500 ml. se mezclan: 125 ml. de formamida desionizada, 125 ml. de 20xSSC, 5 ml. de 10% SDS, 50 mg BSA y 245 ml. de agua destilada) en un coplin por cada dos arrays.
26. Sumergir los arrays durante 15 minutos en el coplin con el tampón de pre-hibridación.
27. Lavar los arrays en un coplin con agua destilada sumergiéndolos de 10 a 12 veces.
28. Secar (con una centrífuga de cristales o en una centrífuga grande a 800 g. durante 4 min).

PREPARACIÓN DE LA SONDA PARA LA HIBRIDACIÓN – DÍA 2

29. Dejar disolver el precipitado de la sonda con el tampón de hibridación durante 30-45 min.
30. Desnaturalizar durante 15 min. a 70ºC.
31. Re-anillamiento durante 30 min. a 37ºC (opcional).

HIBRIDACIÓN – DÍA 2

32. Aplicar los 50 µl. de la mezcla de sondas sobre la línea central de un cubre 22x60 limpio.
33. Colocar el cristal sobre el cubre con cuidado.
34. Emplazar el cristal en una cámara de hibridación con papel científico humedecido con 1 ml. de tampón de lavado 2 (2xSSC, 50% formamida).
35. Hibridar a 37ºC durante 72 h. en oscuridad.

LAVADOS POST-HIBRIDACIÓN – DÍA 3

36. Sumergir el cristal en una placa petri con **tampón de lavado 1** (2xSSC, 0'1%SDS) durante 15 min. a TA y en agitación.
37. Retirar el cubre con cuidado.
38. Sumergir el cristal en una placa petri con **tampón de lavado 2** (2xSSC, 50% formamida) durante 15 min. a 45ºC y en agitación.
39. Sumergir el cristal en un coplin con **tampón de lavado 3** (2xSSC, 0'1%SDS) durante 30 min. a 45ºC y en agitación.
40. Sumergir el cristal en un coplin con **tampón de lavado 4** (0'2xSSC) durante 15 min. a TA y en agitación.
41. Secar (con una centrífuga de cristales o en una centrífuga grande a 800 g. durante 4 min.)

5.1.2. Adquisición de la imagen y análisis de los datos

Las imágenes de los arrays fueron obtenidas mediante un escáner dual GenePix 4000B (Axon Instruments, Downingtown, PA, USA). Los datos de fluorescencia de cada hibridación fueron procesados y analizados con el programa informático GenePix Pro 5.0 (Axon Instruments, Union City, CA, USA) para obtener los $\log_2$ratio (tumor/referencia) de cada cristal.

Acto seguido, se realizó la normalización de los $\log_2$ratio usando la aplicación informática DNMAD (Vaquerizas *et al.*, 2004). Esta herramienta nos permitió combinar y filtrar las réplicas de los clones de cada array y del experimento del marcaje opuesto. Se eliminaron los valores de las réplicas inconsistentes que presentaban un $\log_2$ratio con una diferencia >0'3 con la mediana de los $\log_2$ratio de todas las réplicas. También se desecharon aquellos clones que no presentaran datos en > 70% de los casos analizados.

Finalmente, se utilizó el algoritmo "Binary Segmentation" implementado en la aplicación Insilico CGH (Vaquerizas *et al.*, 2005) para definir las aberraciones en el número de copias. Este algoritmo define segmentos genómicos con unos valores estimativos del número de copias. Cada segmento presenta un valor en $\log_2$ratio igual a la mediana de los $\log_2$ratio de los clones presentes dentro del segmento genómico. Aquellos segmentos con un $\log_2$ratio≥0'1 fueron considerados como ganados, mientras que aquellos con un $\log_2$ratio ≤0'1 se consideraron como perdidos. Los valores fueron categorizados como 0, 1 y -1 para no cambio, ganancia o pérdida, respectivamente. Las regiones genómicas de alteración se definieron como un grupo de al menos dos clones consecutivos que presentaban el mismo valor categorizado de número de copias. Las amplificaciones de alto nivel se consideraron cuando $\log_2$ratio≥0'4.

Para la visualización de los datos, se utilizaron dos programas informáticos: el CGH-Analyzer (Greshock *et al.*, 2004) y el CGH-Explorer (Lingjaerde *et al.*, 2005).

5.1.3. La estandarización de un patrón de artefactos en aCGH

A pesar de los esfuerzos realizados para conseguir un ADN de tejidos en parafina de buena calidad (ver Material y Métodos, 2.2.2), a lo largo del análisis de las muestras de

cáncer de mama, se observó un patrón recurrente de artefactos (ver Resultados 2.1). Este patrón fue descrito previamente en cCGH (Kirchhoff *et al.*, 1998) y observado recientemente en aCGH en una colaboración de nuestro grupo con el Grupo de Citogenética Molecular del CNIO (Blesa *et al.*, en preparación). Este patrón de artefactos lo denominamos "Artifactual-Copy Number Variation" o Ar-CNV y genera unos índices anormales en determinadas regiones cromosómicas, tales como 1p36, 2q37, 4p16, 6p21, 9q34, 11q13 y 12q13 (Figura 11A), que podrían ser considerados erróneamente como ganancias durante el análisis. Obtuvimos también este patrón de artefactos en una serie de hibridaciones de ADN de tejido de mama normal parafinada frente al ADN de referencia. Kirchhoff y cols. describieron un patrón no aleatorio de desviaciones en cCGH de muestras normales y aplicaron un método de estandarización para incrementar la especificidad y la sensibilidad de la técnica, obteniendo con ello un importante descenso en el número de falsos positivos (Kirchhoff *et al.*, 1998). Por consiguiente, para disminuir el impacto de los Ar-CNV en nuestra serie, aplicamos un método de estandarización a toda muestra que presentara el susodicho patrón. Para la estandarización, se restó de los valores de $\log_2$ratio de cada clon el valor de la mediana del $\log_2$ratio de ese clon en la serie de hibridaciones de muestras normales que también presentaban el patrón Ar-CNV. Después de la estandarización, se volvieron a analizar los casos (ver Material y Métodos 5.1.2) y se vio que los patrones de aCGH no presentaban los mencionados artefactos (Figura 11B). Para validar el procedimiento de estandarización, se realizaron pruebas FISH sobre regiones afectadas por los artefactos (ver Material y Métodos 3.4.1) (Figura 11C-D).

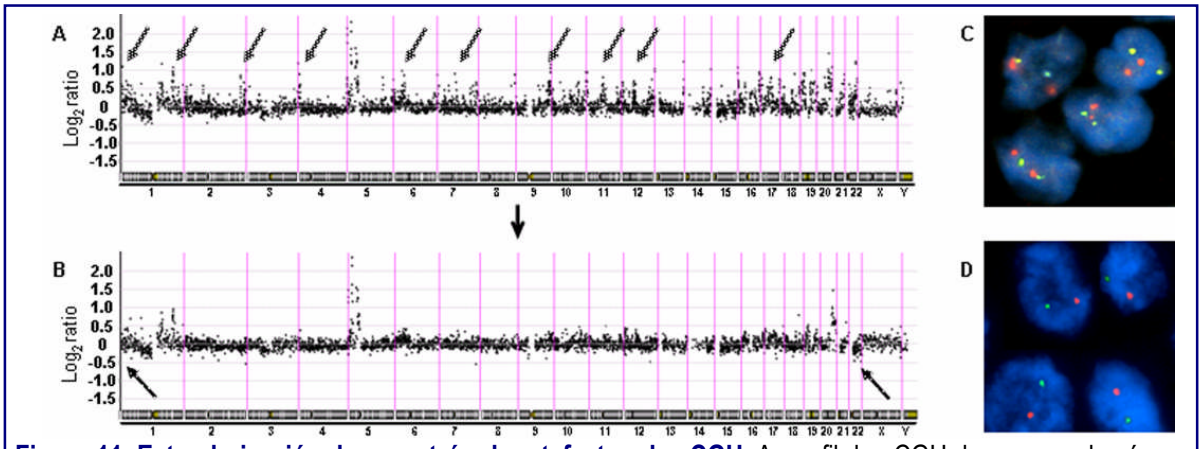


Figura 11. Estandarización de un patrón de artefactos de aCGH. A, perfil de aCGH de un caso de cáncer de mama familiar con artefactos en las regiones cromosómicas señaladas por flechas. B, perfil de aCGH del mismo caso después de la estandarización. Las aberraciones en 1q, 5p ó 20q se consideran verdaderas ganancias dado que se mantienen tras la estandarización. Las flechas por debajo del perfil señalan regiones posteriormente validadas por FISH. C, FISH con la sonda 1p36 que muestra 2 señales rojas (centrómero) y 2 verdes (1p36), lo que apoya el perfil de aCGH tras la estandarización. D, FISH con la sonda del cromosoma 22 que muestra monosomía (1 señal roja y 1 verde), de acuerdo con el perfil de aCGH tras estandarización.

5.2. PLATAFORMA DE ARRAY DE CGH DE LA REGIÓN DE AMPLIFICACIÓN 8p11-p12

Para abordar este proyecto, utilizamos una plataforma de aCGH desarrollada en la Universidad de Cambridge y empleada en estudios anteriores (Garcia *et al.*, 2005; Huang *et al.*, 2004; Pole *et al.*, 2006). El array se diseñó para cubrir con una mayor resolución la región 8p11-p12 con un total de 91 clones BAC, dispuestos desde la posición 31'03 Mb (RP11-473A17) hasta 43'38 Mb (CTD-2115H11). El resto del cromosoma 8 quedaba cubierto por 82 clones dispuestos a 1'5 Mb de resolución. El array también contenía otros 34 clones cubriendo genes relacionados con cáncer y 285 más que cubrían el resto del genoma a una resolución de 10 Mb. Finalmente, seis clones de *Drosophila melanogaster* de la librería RPCI-98 (http://www.chori.org/bacpac) se utilizaron como controles. Los clones que componían el array fueron seleccionados usando el borrador de la secuencia del genoma humano de la Universidad de California (http://genome.ucsc.edu). La mayoría de los clones procedían de la librería RP11, excepto unos pocos procedentes del *Wellcome Trust Sanger Institute* (Hinxton, U.K.) o de Invitrogen (Paisley, U.K.). El ADN de los clones BAC fue aislado usando "micropreps" y amplificados mediante DOP-PCR como se describe previamente (Fiegler *et al.*, 2003), se precipitaron con etanol y se disolvieron en 150mM tampón fosfato a una concentración de ~300 ng/µl. Después, se imprimieron por triplicado en cristales "amine-binding" (CodeLink Activated Slides, Amersham Biosciences, Buckinghamshire, UK) usando un "arrayer MicroGrid II" (BioRobotics, Boston, MA, USA).

5.2.1. Marcaje del ADN e hibridación

Los protocolos de hibridación y marcaje se basaron en los previamente descritos (Fiegler *et al.*, 2003) con leves modificaciones. Los volúmenes fueron calculados para una superficie de *array* de 2x2cm y la prehibridación e hibridación del cristal se realizaron asegurando el área del *array* con un plástico adhesivo. La evaporación del tampón de hibridación se evitó situando los cristales en cámaras húmedas (Camlab Ltd., Cambridge, UK). Los cristales se lavaron con PBS/0,05% Tween20 durante 10 min. a TA antes y después de un lavado con 50% formamida /0,5xSSC durante 30 min. a 42ºC.

5.2.2. Adquisición de la imagen, análisis de los datos y validación del array

Se obtuvieron imágenes de las hibridaciones por medio de un escáner Axon 4100A (Axon Instruments, Union City, CA, USA). Se utilizó el programa informático GenePix Pro v.4.1 (Axon Instruments) para el procesamiento de los resultados y el cálculo de las intensidades después de la sustracción de la señal de fondo. El archivo obtenido se abrió con una plantilla Excel y aquellos puntos con una intensidad menor a dos veces la mediana de la intensidad de los clones de *Drosophila* fueron eliminados. Las relaciones muestra/control se calcularon y normalizaron con la mediana de la relación de los clones de los cromosomas autosómicos. Los clones con unas relaciones con una diferencia mayor del 10% a la mediana de las relaciones de las réplicas también se eliminaron. Si un mínimo de dos puntos por triplicado eran aceptados, se calculó la media de los $\log_2$ ratios y se situó en una gráfica en función de su posición cromosómica en la versión 35 de la secuencia del genoma humano del NCBI.

Se utilizaron líneas celulares previamente caracterizadas por cariotipado de color (SKY) y FISH (Adelaide *et al.*, 2003; Courtay-Cahen *et al.*, 2000; Davidson *et al.*, 2000) para calcular la capacidad del array de detectar cambios en el número de copias. También se incluyeron líneas celulares como GM04626 y GM0141 con tres y cuatro copias del cromosoma X, respectivamente (Coriell Institute for Medical Research). En hibridaciones varón frente a mujer, mujer contra mujer, GM04626 frente a mujer y GM0141 frente a mujer, la media del ratio para los clones del cromosoma X fueron 0’64±0’11, 1’02±0’06, 1’33±0’06 y 1’64±0’09 respectivamente, mientras que la media normalizada para la relación de los clones autosómicos fue 1±0’05. Esto demostró la capacidad del array de describir alteraciones en el número de copias tan pequeñas como un simple cambio. Cuando los límites de ganancia (>1’2, $\log_2$ ratio = 0’26) y de pérdida (<0’8, $\log_2$ ratio = -0’32) fueron utilizados, la tasa de falsos positivos fue <0’2%. Para análisis sucesivos, se adoptaron estos límites para definir ganancias y pérdidas, respectivamente, y un límite superior a 1’75 ($\log_2$ ratio>0’8) para definir amplificación.

Para analizar los resultados e identificar cambios recurrentes entre las muestras se utilizó el programa informático CGH-Analyzer (Greshock *et al.*, 2004).

6. LAS MATRICES DE TEJIDO E INMUNOHISTOQUÍMICA

Una parte importante de esta tesis consistió en la búsqueda de asociaciones entre las aberraciones genómicas estudiadas o un determinado patrón de aberraciones con la expresión de proteínas en el tumor. El análisis de la variación en la expresión proteica de los tumores se hizo en paralelo mediante la construcción de matrices de tejidos y el uso de diferentes anticuerpos.

6.1. LAS MATRICES DE TEJIDO

Se seleccionaron cuidadosamente áreas representativas de cada uno de los tumores mediante observación de secciones completas del tumor teñidas con hematoxilina-eosina. Las zonas seleccionadas se marcaron en los bloques individuales de parafina obteniendo dos cilindros de tejido (1 mm. de diámetro) de cada espécimen. Además, se incluyeron como controles 4 muestras no neoplásicas de tejido mamario y 4 cilindros de tejido de amígdala normal. La unidad de inmunohistoquímica del CNIO construyó las matrices de tejido del siguiente modo: los cilindros del tejido fueron puestos en un orden predeterminado en un nuevo bloque de parafina usando un aparato especifico (Beecher Instruments, Silver Spring, MD) dotado con dos agujas huecas que se desplazan en los dos ejes del plano mediante un sistema manual de rosca calibrado. Una de las agujas sirve para extraer cilindros de parafina del bloque receptor y otra para extraer cilindros del bloque donante, que posteriormente se introducen en el bloque receptor (Kononen *et al.*, 1998). Una sección teñida con hematoxilina-eosina, fue revisada para confirmar la presencia de áreas morfológicamente representativas de las lesiones originales.

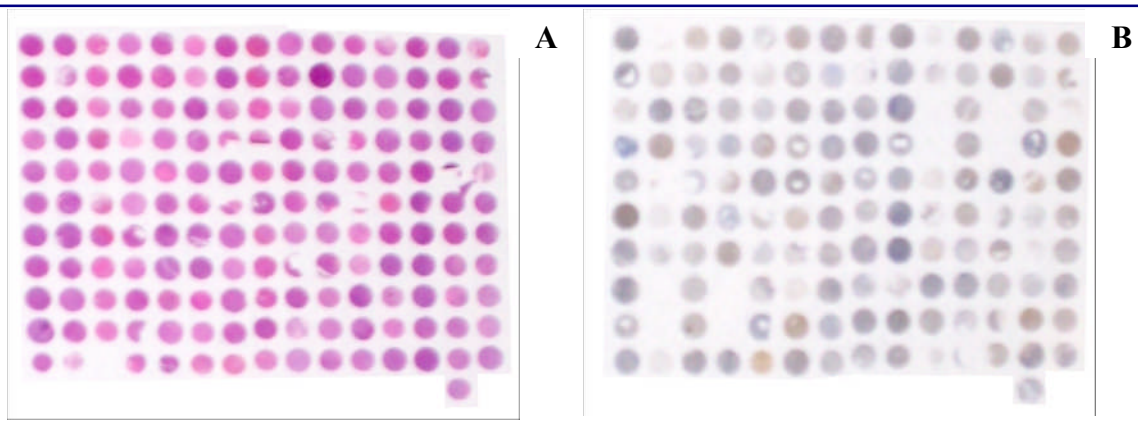


Figura 12. Matriz de tejido. A, teñida con hematoxilina y eosina. B, con inmunohistoquímica.

6.2. INMUNOHISTOQUÍMICA

La inmunohistoquímica (IHQ) es una técnica basada en el uso de un anticuerpo específico, marcado previamente mediante un enlace químico con una sustancia que puede transformarse en visible sin afectar la capacidad del anticuerpo para formar un complejo con el antígeno. Mediante el uso de técnicas específicas, se localiza e identifica el complejo antígeno-anticuerpo dentro de la muestra a estudiar. En las técnicas inmunoenzimáticas, se utilizan como marcadores enzimas capaces de hacer cambiar de color un sustrato incoloro permitiendo una localización más precisa de las reacciones, y ya que la tinción es permanente y estable, se puede contrastar y evaluar con microscopio de luz. Algunas de las enzimas más utilizadas son la peroxidasa y la fosfatasa alcalina, y los sustratos diaminobenzidina (color pardo), aminoetilcarbazol (color rojo) y nitroazul de tetrazolio (color azul).

La IHQ tiene utilidad diagnóstica en identificación de diferenciación y de marcadores pronósticos de neoplasias (marcadores tumorales). Por ejemplo, es posible la identificación de los productos de oncogenes y de genes supresores de tumores con anticuerpos monoclonales. Un elemento importante a considerar es la óptima preservación del tejido y por ende de los antígenos. La mayoría de los antígenos se conservan adecuadamente después de la fijación en formalina e inclusión en parafina. Algunos son más lábiles y sólo se detectan en cortes de congelación.

En nuestro proyecto, la tinción de IHQ fue realizada por el método de Envision (DakoCytomation, Glostrup, Dinamarca) con recuperación antigénica por calor. Las secciones de la matriz de tejido se introdujeron en una estufa a 55ºC toda la noche. Al día siguiente, después de desparafinarlas con xilol y alcoholes de graduación decreciente, fueron sumergidas en citrato sódico a 10mM a pH 6’5 o EDTA a pH 8 (dependiendo del anticuerpo) y hervidas durante 2 min. en una olla a presión. Algunos de ellos, después de pasar por la olla, se incubaron con proteinasa K a temperatura ambiente durante 10 min. En el caso de HER-2, la recuperación antigénica se realizó en baño a 99ºC con citrato sódico a pH 6, durante 40 min. Dos de los anticuerpos, BAX y CDK2, no necesitan desenmascaramiento antigénico y se procedió con la tinción IHQ directamente después de desparafinar. Los anticuerpos, diluciones, casas comerciales y el método de desenmascaramiento están enumerados en la Tabla 3.

Tabla 3. Anticuerpos utilizados.

Anticuerpo	Localización de la tinción	Clon	Dilución	Casa comercial	Punto de corte para χ^2	Desenmascaramiento	Para agrupar subtipos (Sí/No)
RE	N	1D5	1:30	Novocastra	10	CT	S
RP	N	1ª6	1:30	Novocastra	10	CT	S
BCL2	C	124	1:80	DAKO	70	CT	S
Ki-67	N	MIB1	1:30	DAKO	0-5/6-25/>25	CT	S
p53	N	DO-7	1:50	Novocastra	25	CT	S
EGFR	M	EGFR.113	1:10	Novocastra	*	CT	S
HER-2	M	Herceptest	prediluido	DAKO	3+	CT	S
E-caderina	M	4A2C7	1:200	Zymed	†	CT	N
P-caderina	M	56	1:200	Transduction Lab	10	CT	N
B-catenina	M	14	1:1000	Transduction Lab	†	CT	N
γ-catenina	M	15	1:1000	Transduction Lab	†	CT	N
$p120^{ctn}$	M	98	1:500	Transduction Lab	†	CT	N
MDM2	N	IF2	1:10	Oncogene	*	CT	S
Topo IIα	N	Ki-S1	1:400	DAKO	*	CT	S
Ciclina D1	N	DCS-6	1:100	DAKO	40	CT+PK	S
Ciclina D3	N	DCS-22	1:10	Novocastra	*	CT+PK	S
CDK4	N	35.1	1:10	Chemicon	*	CT	N
Ciclina E	N	13A3	1:10	Novocastra	*	CT	S
Ciclina A	N	6E6	1:100	Novocastra	*	CT	S
CDK2	N	8D4	1:500	NeoMarkers	*	S/O	N
Rb	N	G3-245	1:250	BD PharMingen	*	CT	S
E2F6	N	Poly goat	1:50	Santa Cruz	*	CT	S
Ciclina B1	N	7A9	1:25	Novocastra	*	CT	S
CDK1	N	1	1:1500	Transduction Lab	*	CT	N
p16	N	Poly Mouse	1:50	Santa Cruz	60	CT	S
p21	N	EA10	1:50	Oncogene	*	CT	S
p27	N	57	1:1000	Transduction Lab	60	CT	S
SKP2	N	1G12E9	1:10	Zymed	*	CT+PK	S
BAX	C	Poly Rabbit	1:750	Santa Cruz	‡0/1/2/3	S/O	N
BCLXL	C	2H12	1:10	Zymed	‡0/1	CT	N
Survivina	N	Poly Rabbit	1:1000	RD Systems	*	CT	S
NFKB p65	C	F6	1:350	Santa Cruz	‡0/1/2/3	CT	N
Caspasa 3 activa	C	C92-605	1:25	BD PharMingen	*	CT	N
CK 5/6	C y M	D5/16 B4	1:25	DAKO	*	CT	S
CK 8	C y M	35BH11	1:10	DAKO	80	CT	S
CAM 5.2	C y M	CAM 5.2	1:25	Becton Dickinson	80	CT	N
Vimentina	C	V9D	1:500	DAKO	*	CT	S
RAD51	N	51RAD01	1:25	NeoMarkers	*	EDTA	N
RAD50	N	2C6	1:50	Abcam	80	CT	N
XRCC3	N	Poly Rabbit	1:150	NeoMarkers	*	CT	N
ATM	N	Poly Rabbit	1:150	Chemicon	*	CT+PK	N
PCNA	N	PC10	1:5000	Oncogene	80	CT	N
CHEK2	N	DCS-270	1:25	Novocastra	60	EDTA	S
TFDP1	N	1DP06	1:100	NeoMarkers	25	CT	N

* Cualquier célula positiva.
† Expresión membranosa en más del 75% de las células.
‡ Niveles de intensidad ya que la tinción era generalizada.
N (núcleo); M (membrana); C (citoplasma); CT (citrato); PK (proteinasa K); S/O (sin olla).

Para la evaluación de la tinción IHQ de los distintos marcadores, se valoró el porcentaje de células teñidas y posteriormente se eligió un punto de corte como umbral para considerar un caso positivo, que utilizamos después en los análisis estadísticos de Chi-

cuadrado. Estos puntos de corte se eligieron en función de la mediana de los valores o de los puntos de corte previamente publicados para esos mismos marcadores. Los valores de porcentaje se utilizaron como una variable continua para los análisis de agrupamiento. Los siguientes marcadores se trataron de forma excepcional: Ki-67, en el que en lugar de dos niveles (positivo/negativo)establecimos tres niveles de positividad: 0-5%, 6-25% y >25%; HER-2, evaluado según el sistema de cuatro categorías (0 a 3+) propuesto por DAKO para la evaluación del HercepTest; E-caderina y cateninas (β, γ y p120ctn), en las que un tumor se consideró positivo cuando preservaba la expresión, esto es, que al menos el 75% de las células mostraran tinción membranosa completa de intensidad similar al epitelio normal de la mama (Gamallo *et al.*, 1993), el resto de los casos se consideraron como expresión reducida de E-caderina o cateninas; NFKB, BAX y BCLXL, los cuales se analizaron por niveles de intensidad de tinción ya que todas las células eran positivas, estableciendo cuatro niveles para los dos primeros y dos para el tercero. Una descripción más amplia de estos métodos se encuentran en los trabajos previos realizados por nuestro grupo (Honrado *et al.*, 2007; Honrado *et al.*, 2005b; Palacios *et al.*, 2003). En la Tabla 3 se especifica todos los anticuerpos utilizados y la localización de la tinción dentro de la célula, así como el punto de corte, establecido como umbral para considerar un caso positivo, para cada marcador.

7. ESTUDIOS MOLECULARES

7.1. ANÁLISIS DE LOS PATRONES DE METILACIÓN DEL PROMOTOR DE *BRCA1*

Los patrones de metilación del ADN en las islas CpG del gen *BRCA1* fueron determinados por PCR específica para metilación (Herman *et al.*, 1996). Esta técnica distingue alelos no metilados de metilados en un gen en base a los cambios en la secuencia producidos después del tratamiento del ADN con bisulfito, que convierte las citosinas en uracilos en los genes no metilados, pero no en los metilados. Después, mediante PCR utilizando cebadores diseñados específicamente para que se unan al ADN metilado o no metilado, obtenemos un producto de 86 pb. en el caso de que el gen no esté metilado y de 75 pb. si el gen está metilado. La secuencia de los cebadores de *BRCA1* para la reacción de ADN no metilado son 5´-TTG GTT TTT GTG GTA ATG GAA AAG TGT-3´ (sentido, S) y de 5´-CAA AAA ATC TCA ACA AAC TCA CAC CA-3´ (antisentido, AS) y para la reacción de ADN metilado 5´-TCG TGG TAA CGG AAA AGC GC-3´ (S) y 5´-AAA TCT CAA CGA ACT CAC GCC G-3´ (AS). El cebador S de la reacción del ADN no metilado comienza a 1536 pb., y el cebador AS de la reacción del ADN metilado comienza a 1543 pb. de la secuencia U37574 del Gen-Bank. Se utilizaron como controles positivo y negativo, ADN de placenta tratada *in vitro* con la metilasa bacteriana SssI y ADN de linfocitos normales, respectivamente. Se cargaron 10 µl. de cada reacción de PCR directamente sobre geles no desnaturalizantes de poliacrilamida al 6%, teñidos con bromuro de etidio, y se visualizaron bajo iluminación UV.

7.2. ANÁLISIS DE LOH DE *BRCA1* MEDIANTE MICROSATÉLITES

En cada paciente, se extrajo ADN de sangre y de tumor y ambos se analizaron para las pérdidas alélicas mediante los marcadores microsatélites D17S1322, y D17S855, localizados en los intrones 19 y 20, respectivamente del gen *BRCA1,* en la región 17q21 (Smith *et al.*, 1996) y el D17S1327 que se localiza en 17q21 pero fuera del gen *BRCA1*. Los marcadores fueron elegidos basados en su alta heterocigosidad, previamente publicada (Phelan *et al.*, 1998). El cebador sentido para cada microsatélite fue marcado con fluorescencia: FAM (Applied Biosystems/ PE Biosystems, Foster city, California, USA). En todos los casos, los marcadores fueron evaluados inicialmente en el ADN de linfocitos y los marcadores informativos fueron estudiados en el tejido del tumor. Los ciclos de la

reacción de PCR fueron los siguientes: 95°C 5 min., 35 ciclos a 94°C 60 segundos, 55°C 60 segundos, y 72°C 90 segundos, seguido del alargamiento final a 72°C 5 min. Los tamaños del alelo fueron determinados mediante el secuenciador capilar ABI Prism™ 310 (Applied Biosystems, Perkin Elmer, Warrington, UK). Las señales fluorescentes de los alelos con diversos tamaños fueron registradas y analizadas usando el software de la versión 3.1 de GeneScan (Applied Biosystems, Warrington, UK). Se consideró LOH cuando las diferencias entre el pico del tumor y de los linfocitos normales era mayor del 25%.

8. ANÁLISIS ESTADÍSTICOS

Todos los resultados se sometieron a diferentes análisis estadísticos para una oportuna interpretación de los mismos y para la descripción de asociaciones.

8.1. ANÁLISIS DE LOS DATOS GENÓMICOS

Para distinguir si un grupo de casos presentaba un mayor o menor número de cambios genómicos por caso, se usó la prueba no paramétrica de U de Mann-Whitney utilizando el programa estadístico SPSS para Windows (SPSS Inc. Chicago, IL, USA).

Por otro lado, las diferencias en las frecuencias de aberración por región cromosómica fueron analizadas mediante la herramienta POMELO (Herrero *et al.*, 2003), disponible en http://pomelo.bioinfo.cnio.es. Esta herramienta permite el análisis múltiple de las frecuencias de aberración por cada caso y cada grupo de casos previamente establecidos. Debido al gran número de hipótesis que se plantean (tantas como regiones incluidas), esta no es una tarea trivial. El POMELO está diseñado para controlar el problema de los análisis múltiples incluyendo cuatro métodos para ajustar la probabilidad (p): dos que controlan el "Family Wise Error Rate" y dos que controlan el "False Discovery Rate". Dado que previamente se habían establecido valores discretos para definir ganancias, no cambios o pérdidas (Material y Métodos 4.4 y 5.1.2), de las cinco pruebas estadísticas disponibles en el POMELO, se empleó la prueba exacta de Fisher con un ajuste de la probabilidad para análisis múltiples basado en el "False Discovery Rate", para los datos procedentes de los estudios con aCGH; no así para el análisis de los datos de cCGH, en los que la prueba de Fisher se realizó sin el ajuste para análisis múltiple.

8.2. ANÁLISIS DE AGRUPAMIENTO JERÁRQUICO NO SUPERVISADO

Se realizaron análisis de agrupamiento jerárquico no supervisado con variables discretas procedentes de los estudios genómicos para observar el agrupamiento de los tumores en función de sus aberraciones genómicas. Para ello se emplearon métodos de correlación incluidos en el programa informático CLUSTER (Eisen *et al.*, 1998).

Por otra parte, también se llevaron a cabo análisis de agrupamiento no supervisado para agrupar los tumores en función de su patrón de IHQ a partir de los datos del

porcentaje de células positivas para cada marcador de IHQ. Los datos se utilizaron sin establecer niveles para considerar un tumor positivo. En el caso de grado, BAX, BCLX, NFKB p65 introdujimos 0, 1, 2, 3 como 0%, 33%, 66% y 100%. Los valores de las moléculas de adhesión y de HER-2 fueron introducidos como 100% en aquellos tumores que estaban conservados o eran positivos para HER-2 y el 0% cuando estaban reducidos o eran negativos respectivamente. Para el análisis de agrupamiento jerárquico no supervisado se empleó la aplicación GEPAS, disponible en línea en la página http://gepas.bioinfo.cnio.es/ (Herrero *et al.*, 2003; Herrero *et al.*, 2001). Concretamente, se empleó el algoritmo SOTA ("Self-Organizing Tree Algorithm"), el cual es una red neural no supervisada con una topología de árbol binario que permite el agrupamiento de las muestras en función de los perfiles de expresión o de aberraciones genómicas y, a su vez, de los genes que tienen perfiles similares. Este paquete permite la utilización de varios coeficientes para calcular la distancia en los agrupamientos (tanto de las muestras como de los genes). Los dendrogramas de las muestras se realizaron con la distancia UPGMA euclídea y para las distancias de los genes se utilizó el coeficiente de correlación lineal. Las distancias euclídeas agrupan los patrones dependiendo de los niveles de activación o represión, mientras que las distancias de correlación lineal agrupan patrones con tendencias similares.

8.3. ESTUDIOS DE ASOCIACIÓN CON IHQ

Finalmente, para determinar las diferencias IHQ que presentaban los diferentes grupos establecidos a lo largo de los análisis, se usó la prueba de chi cuadrado con la corrección de la prueba exacta de Fisher cuando era necesaria, siguiendo los niveles establecidos para considerar un caso como positivo o negativo (Tabla 3). El programa estadístico SPSS para Windows (SPSS, Inc., Chicago, IL) fue utilizado a tal efecto.

RESULTADOS

A continuación se muestran los resultados cosechados a lo largo de la presente tesis desde el año 2003 hasta la redacción de la misma (finales de 2007). Como mencionamos en el primer epígrafe de Material y Métodos, se han abordado un total de cinco estudios combinando técnicas tales como la cCGH, el aCGH, la FISH y la IHQ sobre una serie variable de muestras. Estos análisis se van a ver desglosados a lo largo de la presente sección. A modo de resumen:

El primer estudio se centra en los patrones de amplificación por cCGH de 80 muestras de cáncer de mama familiar y su asociación con diferentes parámetros clínicos e IHQ (Melchor *et al.*, 2005).

El segundo análisis es el primer estudio por aCGH de muestras de cáncer de mama familiar abordado por nuestro grupo y, aparte de describir los patrones de aberraciones genómicas por clase de cáncer de mama en un total de 19 *BRCA1*, 24 *BRCA2*, 32 BRCAX y 19 tumores esporádicos), pone a prueba un clasificador de muestras previamente publicado (Jonsson *et al.*, 2005) y señala la importancia del receptor de estrógenos en el perfil de aberraciones genómicas (Melchor *et al.*, 2007c).

El tercer estudio se basa en la clasificación por IHQ de muestras de cáncer de mama familiar (18 *BRCA1*, 16 *BRCA2*, 28 BRCAX) en una serie de subtipos moleculares que se asemejan a los descritos con anterioridad en cáncer de mama esporádico. Asimismo, describimos los diferentes patrones de aberraciones genómicas y de amplificaciones asociados a cada uno de estos subtipos (Melchor *et al.*, 2007b).

El cuarto y quinto estudio se enfocaron en los análisis de caracterización genómica de dos regiones de amplificación y de su posible asociación con parámetros clínicos e IHQ. Las regiones estudiadas fueron: 8p11-p12 (Melchor *et al.*, 2007a) y 13q34.

RESULTADOS PARTE I

La acumulación de amplificaciones específicas caracteriza dos rutas genómicas diferentes de evolución del cáncer de mama familiar

En el cáncer de mama, se han descrito frecuentemente amplificaciones de ADN de alto nivel y algunas de ellas se han asociado con un peor pronóstico. Para determinar la frecuencia y co-ocurrencia de estos eventos genómicos en el cáncer de mama familiar, hemos analizado 80 tumores familiares (26 BRCA1*, 18* BRCA2 *y 36 BRCAX) usando cCGH de alta resolución. Identificamos un total de 21 amplificaciones recurrentes tales como 8q21-q23 (26'25%), 17q22-q25 (13'75%), 13q21-q31 (12'50%) y 8q24 (11'25%), muchas de las cuales fueron comunes a todas las clases familiares. Estas amplificaciones definieron un fenotipo genómico "amplificador" asociado a una mayor inestabilidad genómica. Basándonos en la co-ocurrencia de estas amplificaciones, identificamos dos rutas genómicas de amplificación asociadas con 8q21-q23 y/o 17q22-q25, y con 13q21-q31, respectivamente. Estas rutas se asociaron con otras aberraciones genómicas, con características inmunohistoquímicas asociadas a una mayor o menor agresividad y con una tendencia a asociarse a* BRCA1 *y a* BRCA2*/X, respectivamente. En conclusión, nuestros datos sugieren la presencia de dos patrones diferentes de evolución, que probablemente sean comunes al cáncer de mama esporádico y familiar.*

1. ESTUDIO DE cCGH DE MUESTRAS DE MAMA: PATRÓN ESPECÍFICO DE AMPLIFICACIONES

El primer estudio que compuso la presente tesis tuvo como objeto el análisis de 80 muestras de cáncer de mama familiar (26 *BRCA1*, 18 *BRCA2* y 36 BRCAX) por cCGH de alta resolución (HR-CGH). Este análisis representaba la continuación de un estudio previo realizado por nuestro grupo (Alvarez *et al.*, 2005) con la adición de nuevas muestras y con el objetivo de describir el patrón de amplificaciones de alto nivel que los tumores de mama familiar podrían presentar. Al compartir 72 tumores en ambos estudios, parte de los resultados coincidieron.

El número de cambios genómicos fue mayor en los tumores *BRCA1* y *BRCA2* que en los tumores BRCAX, indicando la mayor inestabilidad genómica que estos tumores podrían tener al presentar mutaciones en genes implicados en la reparación del ADN. Tras el análisis de las 63 regiones cromosómicas previamente establecidas para definir el patrón de HR-CGH (Alvarez *et al.*, 2005), cuatro regiones cromosómicas se describieron comúnmente alteradas en más del 50% de cada uno de los tres grupos de tumores: ganancias en 1q y 8q21-q23, y pérdidas en 8p21-p23 y 11q22-q25 (Tabla 4). Estas aberraciones genómicas podrían representar un conjunto de alteraciones cruciales para el desarrollo del cáncer de mama. Por otra parte, también se registraron una serie de cambios genómicos significativamente más frecuentes en ciertos grupos de tumores tras la comparación estadística entre grupos (P<0’05, prueba exacta de Fisher sin ajustar) (Tabla 4).

1.1. IDENTIFICACIÓN DE REGIONES CON AMPLIFICACIONES DE ALTO NIVEL RECURRENTES

Se registraron un total de 21 regiones cromosómicas recurrentemente afectadas con amplificaciones de alto nivel (HR-CGH ratio>1’5). Consideramos regiones de amplificación recurrente cuando una región presenta amplificación de alto nivel en dos o más casos. Las regiones cromosómicas más frecuentemente alteradas fueron: 8q21-q23 (21 de los 80 casos analizados), 17q22-q25 (11 casos), 13q21-q31 (10 casos), 8q24 (9 casos), 12q15-q21 (7 casos) y 20q12-q13, 15q22-q26 y 3q25-q26.3 (presentes en 6 casos cada uno) (Figura 13).

Tabla 4. Frecuencia de aberraciones genómicas registradas en las 63 regiones cromosómicas del patrón de HR-CGH.

Regiones cromosómicas	Ganancias (%)			Pérdidas (%)			*P*-valor		
	BRCA1	BRCA2	BRCAX	BRCA1	BRCA2	BRCAX	*P*1	*P*2	*P*3
1p	38	28	22	15	17	14			
1q	**65**	**61**	**78**	0	0	3			
2p22-25	12	0	3	23	50	33			
2p11-21	15	0	8	4	28	3	**0'034**		**0'013**
2q11-21	0	0	3	23	28	28			
2q22-34	31	56	22	12	17	14			**0'030**
2q35-37	12	6	6	23	28	22			
3p22-26	8	11	6	27	22	25			
3p14-21	4	6	8	42	39	17		**0'022**	
3p11-13	12	39	19	27	17	17			
3q11-23	12	33	8	23	11	6			**0'047**
3q24-29	50	44	31	4	6	6			
4p15-16	0	0	0	65	28	39	**0'030**		
4p11-14	15	39	17	12	11	14			
4q11-28	35	61	31	27	22	19			**0'042**
4q31-35	19	11	11	50	39	39			
5p14-15.3	8	17	11	31	28	14			
5p11-13	27	33	33	0	6	0			
5q11-23	19	33	19	58	22	31	**0'030**	**0'040**	
5q31-35	4	17	17	62	33	36			
6p	31	28	22	23	6	6			
6q11-24	35	44	25	19	17	25			
6q25-27	0	0	6	46	44	25			
7p	23	17	11	19	11	14			
7q11-31	38	28	36	19	6	8			
7q32-36	15	0	8	31	33	22			
8p21-23	0	0	3	**73**	**83**	**72**			
8p11-12	19	50	28	19	0	11	**0'049**		
8q11-13	31	61	33	8	0	8			
8q21-23	**77**	**78**	**47**	0	0	8		**0'034**	**0'043**
8q24	69	67	31	4	6	8		**0'004**	**0'019**
9p	15	6	3	31	39	42			
9q11-33	8	6	3	38	22	25			
9q34	31	28	14	27	6	11			
10p	23	17	11	15	22	6			
10q	15	6	6	58	56	33			
11p15	0	0	3	23	33	28			
11p11-14	12	28	17	27	22	19			
11q11-21	50	44	33	4	11	8			
11q22-25	4	0	0	**69**	**83**	**69**			
12p	19	11	11	19	28	25			
12q11-21	27	67	37	27	6	8	**0'014**		**0'045**
12q22-24.3	15	6	8	42	22	22			
13q11-14	0	0	0	42	44	36			
13q21-31	19	33	19	42	28	28			
13q32-34	15	11	8	46	39	31			
14q11-23	19	17	14	12	11	17			
14q24-32	4	6	0	54	67	33			**0'040**
15q11-21	8	11	3	54	28	31			
15q22-26	15	17	8	42	11	36	**0'043**		
16p	12	6	25	27	6	14			
16q	0	0	3	46	50	36			
17p	0	0	0	35	28	42			
17q11-21	15	39	31	8	0	17			
17q22-25	65	61	31	0	0	11		**0'010**	**0'042**
18p	12	11	3	15	0	11			
18q	4	6	11	58	11	36	**0'002**		
19p	42	61	42	8	0	0			
19q	27	33	33	8	0	0			
20p	4	6	14	31	11	17			
20q	19	22	31	15	0	6			
21q	8	17	8	27	28	22			
22q	23	28	11	23	22	17			

Las celdas sombreadas indican regiones comúnmente alteradas en todos los tumores BRCA. Se representan solo los *P*-valor<0.05 obtenidos a partir de la prueba exacta de Fisher sin ajustar para test múltiple. P1= *BRCA1* vs *BRCA2*, P2= *BRCA1* vs BRCAX, P3= *BRCA2* vs BRCAX.

Estas regiones frecuentemente amplificadas fueron comunes a todos los grupos BRCA, salvo las amplificaciones de 17q22-q25 y 20q12-q13, que no estaban presentes en las muestras *BRCA2* (Figura 13).

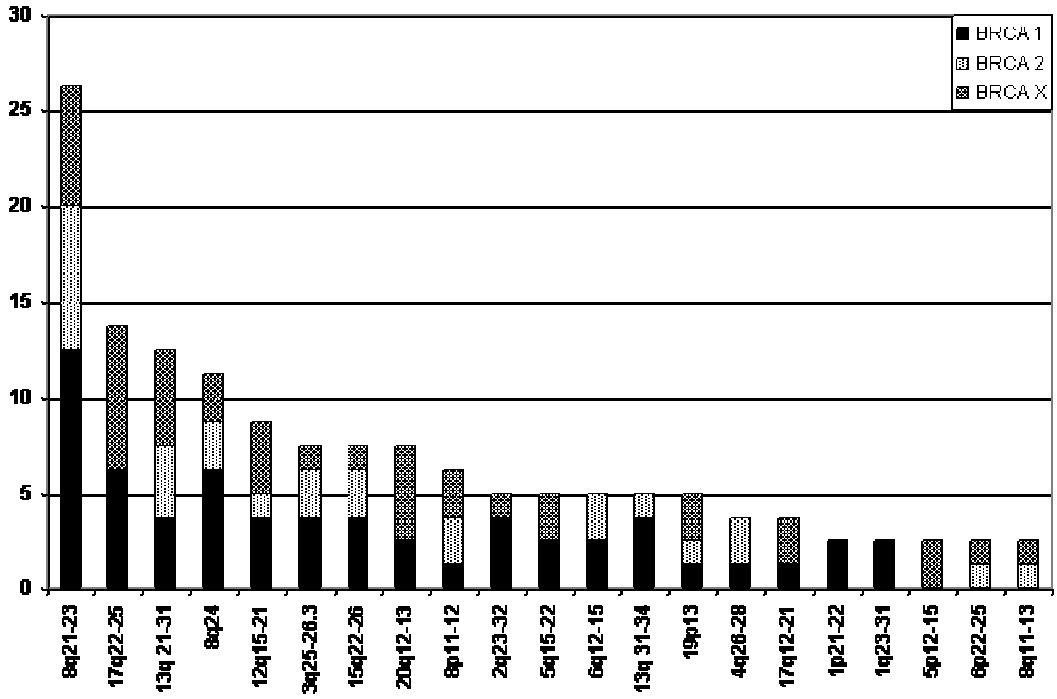


Figura 13. Frecuencia y distribución de las 21 regiones cromosómicas recurrentemente alteradas con amplificación en las 80 muestras de cáncer de mamafamiliar estudiadas.

Cuando nos interesamos por la co-ocurrencia de estas amplificaciones en los tumores, observamos que aproximadamente el 50% de todos los casos (39 muestras) no tenían amplificaciones. Denominamos a este grupo de casos tumores “No-Amplificadores” (NA), en contraposición al resto de muestras que presentaban al menos una amplificación. Para establecer dos categorías diferentes entre los tumores que amplificaban un mayor número de regiones y los que amplificaban un menor número, calculamos la mediana del número de amplificaciones. De esta manera, definimos dos grupos: tumores “Levemente Amplificadores” (LA) que eran casos con una o dos regiones con amplificación, y tumores “Altamente Amplificadores” (AA) que presentaban más de dos regiones afectadas con amplificación. La distribución de estas categorías entre las distintas clases de tumores BRCA se muestra en la Figura 14. Los tumores *BRCA1* parecieron tener una mayor tendencia a la acumulación de amplificaciones que los tumores *BRCA2* ó *BRCAX*, aunque las diferencias que se registraron entre los tres grupos no resultaron ser significativas.

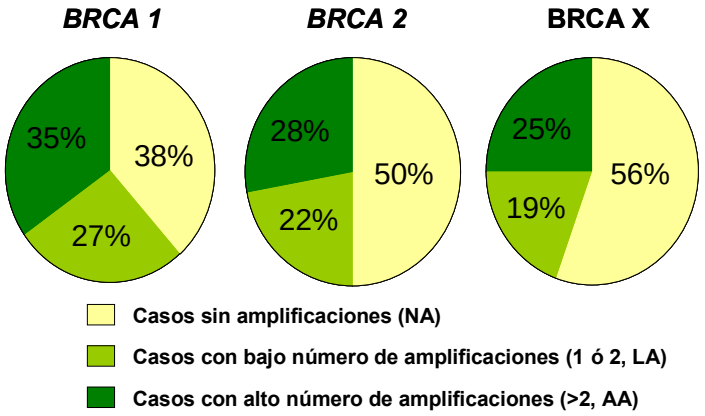


Figura 14. Distribución de los tres fenotipos genómicos descritos en este estudio dentro de las distintas clases de tumores BRCA.

Como cabía esperar, los tumores AA o LA portaban a su vez de forma significativa un mayor número de cambios genómicos que los tumores NA (*P*<0'05, Test de la U de Mann-Whitney) (Figura 15), independientemente de la clase BRCA y de si se consideran las regiones afectadas por amplificación.

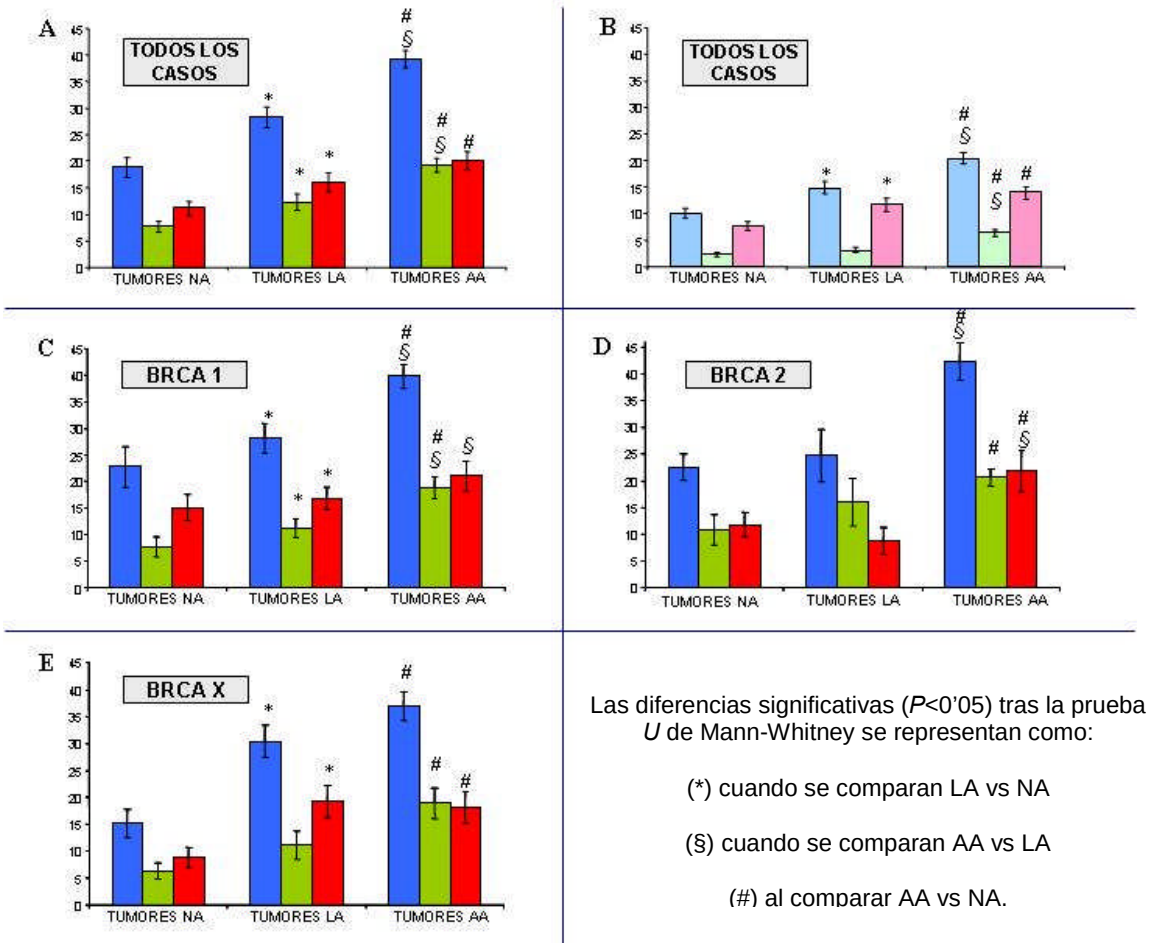


Figura 15. Número de cambios genómicos en los grupos tumorales. Se representa la media del número de cambios totales (azul), ganancias (verde) y pérdidas (rojo) en las 63 regiones cromosómicas por caso de cáncer de mama (1A), por caso BRCA1 (1C), por caso BRCA2 (1D), y por caso BRCAX (1E), categorizados por el número de amplificaciones en tumores NA, LA y AA. 1B, idéntica representación sin tener en cuenta las regiones afectadas por amplificación.

1.2. LA EXISTENCIA DE DOS DIFERENTES RUTAS DE EVOLUCIÓN GENÓMICA

1.2.1. Asociación con amplificaciones específicas

Con el objetivo de ver si existía alguna asociación entre las amplificaciones que los tumores portaban, realizamos un agrupamiento no supervisado de los casos con los datos de las regiones de amplificación (Figura 16). Se separaron dos ramas principales, una de ellas asociada a la amp13q21-q31 que a su vez presentaba una tendencia a acumular otras amplificaciones concretas como 6q12-q15, 5q15-q22, 2q23-q32 o 4q26-q28 (rama naranja); y una segunda rama asociada a la amp8q21-q23 y/o amp17q22-q25, con otras amplificaciones como 15q22-q26 u 8q24 en el grupo de la amp8q21-q23, y la región 20q12-q13 en el grupo amp17q22-q25 (rama verde). Cuando se compararon las frecuencias de amplificación de las regiones entre ambas ramas, observamos diferencias significativas (con valores *P* ajustados<0'05) para 13q21-q31 (*P*=0'000), 8q21-23 (*P*=0'0017) y 6q12-15 (*P*=0'017) (datos no mostrados). Otros cambios genómicos (ganancias y pérdidas) también se distribuían de un modo significativamente diferente entre ambas ramas (datos no mostrados). Cuando se analizó la distribución de los tres grupos BRCA, observamos que estaban distribuidos al azar entre las dos ramas, si bien la mayoría de los tumores *BRCA1* se asociaban con la rama de amp8q21-q23 y/o 17q22-q25; y los tumores *BRCA2*/X con la rama de amp13q21-q31 (Figura 16). Estos resultados apoyarían la existencia de dos rutas genómicas diferentes de evolución a través de distintos cambios cromosómicos.

1.2.2. Asociación con marcadores inmunohistoquímicos

Para determinar si había características inmunohistoquímicas que se asociasen a las regiones de amplificación, definimos el patrón de IHQ analizando parámetros histológicos y varios marcadores de proliferación (Ki67), receptores hormonales (RE y RP), ciclo celular (p53), adhesión celular (p120^{ctn}, Caderina E, Caderina P y γ–catenina), y de apoptosis (BCL2). Se realizaron comparaciones estadísticas entre los grupos (de amp8q21-q23 y/o 17q22-q25; de amp13q21-31; y los No Amplificadores) en busca de algún marcador significativamente diferente (Tabla 5). Los tumores con la amp8q21-q23 y/o 17q22-q25 se caracterizaron por un mayor grado y un mayor número de células en mitosis, alta expresión de Ki67, y tinción negativa para p120^{ctn} y caderina E. Por el contrario, los casos con la amp13q21-q31 presentaban principalmente un bajo grado y número de

mitosis, un bajo nivel de Ki67, tinción positiva para receptores hormonales (RE y RP) en casi el 100% de los casos, tinción negativa para p53 y $p120^{ctn}$, y positiva para caderina E; este último marcador difería significativamente de los tumores NA. Además, los tumores NA presentaron un patrón IHQ similar al de los casos con amp13q21-31, aunque con un menor porcentaje de casos positivos para receptores hormonales y negativos para $p120^{ctn}$ (Tabla 5).

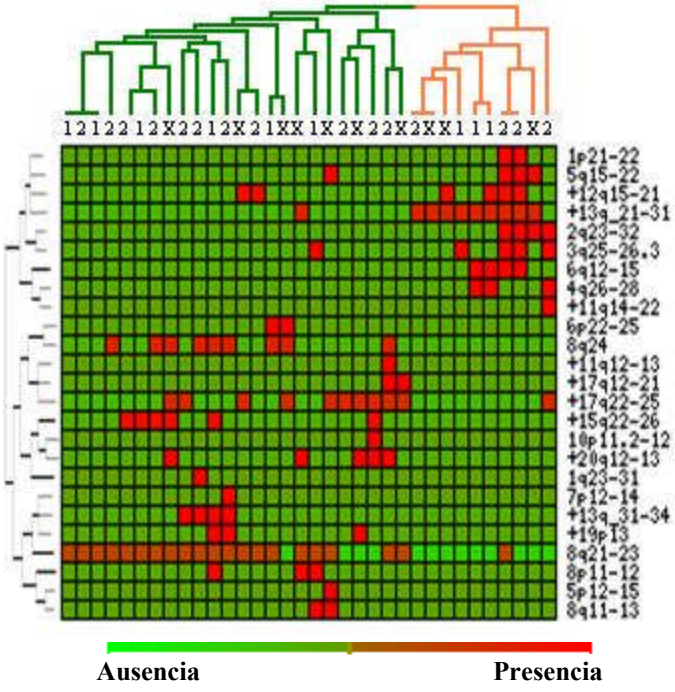


Figura 16. Agrupamiento no supervisado de los casos con las regiones de amplificación. Se representan los 34 casos con al menos una amplificación en las regiones 8q21-q23, 13q21-q31, ó 17q22-q25. Se incluyeron 25 regiones de amplificación (21 recurrentes y 4 no recurrentes). 1, 2 y X representan el grupo BRCA de los tumores. Rojo y verde indican respectivamente presencia y ausencia de amplificación. La rama naranja engloba casos con amp13q21-q31 y otras secundarias, mientras que la rama verde incluye los casos con amp8q21-q23, con o sin la amp17q22-q25 y otras amplificaciones secundarias.

Usando los parámetros IHQ estudiados, hicimos un agrupamiento no supervisado con todos los casos para observar si estos tumores se separaban nítidamente en varios grupos (Figura 17). Describimos dos ramas principales: una asociada con un mayor grado y mitosis, un mayor nivel de Ki67, negativo para receptores hormonales y BCL2, y positivo para p53 (rama derecha); mientras que la segunda rama mostraba un fenotipo opuesto con un menor grado y mitosis, menor expresión de Ki67, positivo para receptores hormonales y BCL2, y negativo para p53 (rama izquierda). La primera rama contenía fundamentalmente a tumores NA-*BRCA1*, y la mayoría de los casos con amp8q21-23 y/o 17q22-25 (18 casos) independientemente del tipo BRCA. En la rama de la izquierda había una mezcla de tumores NA-*BRCA2*/X (23 casos), con amp13q21-31 (5 casos) y con amp8q21-23 (5 casos).

Tabla 5. Comparación de variables de IHQ entre los tumores de mama familiar con amplificación en 8q21-23 y/o 17q22-25, amplificación en 13q21-31, y tumores sin amplificación.

	Casos con amp8q21-q23 y/o 17q22-q25		Casos con amp13q21-q31		Casos No Amplificadores	
	n (%)	P^1	n (%)	P^2	n (%)	P^3
Grado						
1	2 (9'1)		1 (11'1)		13 (37'1)	
2	6 (27'3)	NS	4 (44'4)	NS	9 (25'7)	0'028
3	14 (63'6)		4 (44'4)		13 (37'1)	
Mitosis						
1	3 (15'0)		4 (44'4)		20 (55'6)	
2	4 (20'0)	NS	1 (11'1)	NS	4 (11'1)	0'006
3	13 (65'0)		4 (44'4)		12 (33'3)	
Ki67						
0 – 5%	7 (30'4)		5 (62'5)		24 (64'9)	
6 – 25%	9 (39'1)	NS	2 (25'0)	NS	7 (18'9)	0'040
>25%	7 (30'4)		1 (12'5)		6 (16'2)	
ER						
Negative	11 (47'8)	0'028	0	NS (0'085)	14 (36'8)	NS
Positive	12 (52'2)		8 (100)		24 (63'2)	
PR						
Negative	14 (60'9)	0'037	1 (12'5)	NS (0'115)	18 (47'4)	NS
Positive	9 (39'1)		7 (87'5)		20 (52'6)	
p53						
Negative	16 (69'6)	NS	7 (87'5)	NS	28 (75'7)	NS
Positive	7 (30'4)		1 (12'5)		9 (24'3)	
$p120^{CTN}$						
Negative	15 (78'9)	NS	8 (100)	0'013	16 (50'0)	0'023
Positive	4 (21'1)		0		16 (50'0)	
E – Cadherin						
Negative	12 (60'0)	NS	3 (37'5)	NS	12 (36'4)	0'047
Positive	8 (40'0)		5 (62'5)		21 (63'6)	
P – Cadherin						
Negative	16 (80'0)	NS	8 (100)	NS	26 (81'3)	NS
Positive	4 (20'0)		0		6 (18'8)	
G- Catenin						
Negative	13 (72'2)	NS	6 (75'0)	NS	25 (78'1)	NS
Positive	5 (27'8)		2 (25'0)		7 (21'9)	
BCL2						
Negative	14 (63'6)	NS	5 (62'5)	NS	22 (59'5)	NS
Positive	8 (36'4)		3 (37'5)		15 (40'5)	

Todos los P-valores fueron obtenidos con el test χ^2 de contingencia con la corrección de la prueba exacta de Fisher cuando era necesario. P-valores inferiores a 0.05 fueron considerados como significativos.
P^1 obtenido por comparación de casos con la amp8q/17q vs amp13q.
P^2 obtenido por comparación de casos con la amp13q vs Tumores No Amplificadores.
P^3 obtenido por comparación de casos con la amp8q/17q vs Tumores No Amplificadores.
NS: diferencias no significativas.

En conclusión, lo que este estudio puso de manifiesto fue la existencia de diferentes rutas de amplificación en cáncer de mama familiar y la asociación de una de ellas (la amp8q21-q23 y/o 17q22-q25) a una peor evolución de la enfermedad.

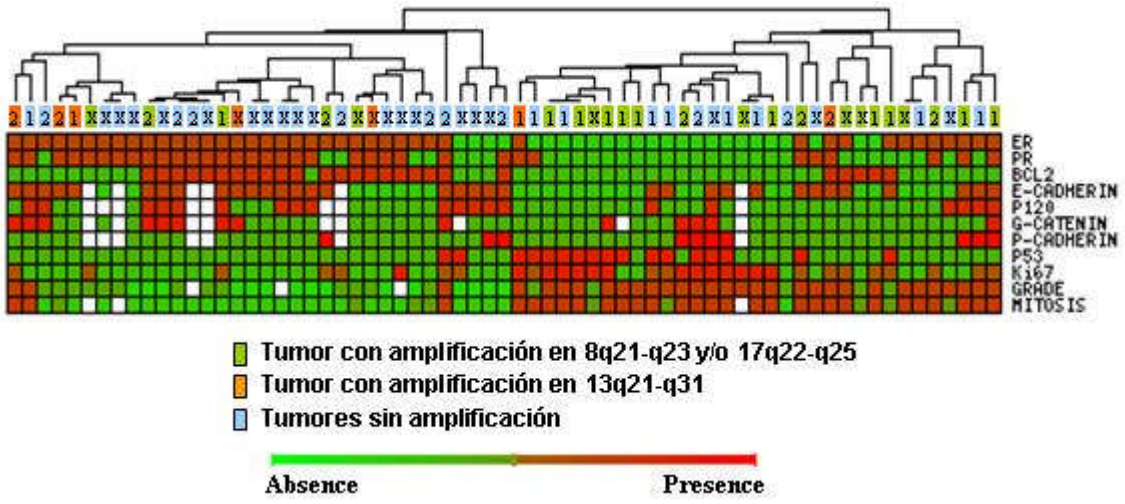


Figura 17. Agrupamiento no supervisado con los marcadores de IHQ en 66 casos. Los casos amplificados sin la amp8q21-23, 13q21-31 ó 17q22-25 no fueron tenidos en cuenta. Valores en rojo indican expresión, en verde ausencia de expresión, y la intensidad es una función del grado de expresión. En blanco, valores no evaluados. La rama derecha presenta marcadores asociados con agresividad, proliferación y amplificación de 8q21-23; la rama izquierda incluye características de baja agresividad, buen pronósticos, amplificación de 13q21-31, y Tumores No Amplificadores.

RESULTADOS PARTE II

El estado del receptor de estrógenos podría modular el patrón de aberraciones genómicas en cáncer de mama familiar y esporádico: nuestro primer estudio basado en aCGH

El cáncer de mama familiar representa el 5-10% de los tumores de mama. Las mutaciones en los dos genes de alta susceptibilidad al cancer de mama, BRCA1 *y* BRCA2*, solo representan una pequeña proporción de estas familias (30%), mientras que la mayoría restante (70%) no presenta mutaciones en estos genes (BRCAX). Para caracterizar y definir las diferencias genómicas entre estas tres clases de tumores familiares y el cáncer de mama esporádico, hemos analizado 19 tumores* BRCA1*, 24* BRCA2 *y 31 BRCAX, así como 19 tumores esporádicos utilizando una plataforma de hibridación genómica comparativa con clones BAC impresos en un cristal y cubriendo el genoma a una resolución de 1 Mb (aCGH). Como resultados, obtuvimos que los tumores* BRCA1/2 *mostraban una mayor inestabilidad genómica que los BRCAX y esporádicos y describimos un conjunto de aberraciones genómicas comunes a todos los grupos de cáncer de mama (tales como +1q, +16p, -8ptel-p12 y -16q. También observamos que la presencia o ausencia del receptor de estrógenos (RE) podría jugar un papel importante en el desarrollo tumoral a través de diferentes rutas genómicas, independientemente del tipo tumoral (familiar o esporádico) y de la mutación* BRCA *(*BRCA1 *ó* BRCA2*). De este modo, los tumores RE-negativos mostraron una mayor inestabilidad genómica y un patrón de aberraciones genómicos considerablemente diferente a los tumores RE-positivos. El papel de los genes* BRCA *(y principalmente* BRCA1*) podría estar enfocado en el patrón de aberraciones genómicas, ya sea incrementando o modulando la inestabilidad genómica.*

2. PRIMER ESTUDIO DE ARRAY-CGH: EL RECEPTOR DE ESTRÓGENOS COMO MARCADOR DEL PATRÓN GENÓMICO EN CÁNCER DE MAMA FAMILIAR Y ESPORÁDICO

El primer análisis de las muestras de cáncer de mama familiar y esporádico con el uso de la plataforma de aCGH tenía como objetivos: 1) establecer el patrón genómico de cada una de las clases de cáncer de mama (tumores asociados a mutación *BRCA1*, tumores asociados a mutación *BRCA2*, tumores familiares no-*BRCA1/2* ó BRCAX y tumores esporádicos); 2) identificar regiones comúnmente alteradas en todas las clases y aquellas aberraciones específicas de clase; y finalmente 3) validar un clasificador de muestras *BRCA1/2* construido usando la misma plataforma de aCGH y publicado durante el inicio de este estudio (Jonsson *et al.*, 2005).

2.1. ESTANDARIZACIÓN DE UN PATRÓN ARTEFACTUAL DE CAMBIOS GENÓMICOS

Se hibridó el ADN tumoral de un total de 93 muestras de cáncer de mama: 19 *BRCA1*, 24 *BRCA2*, 31 BRCAX y 19 esporádicos. Como se mencionó en Material y Métodos 5.1.3, observamos un patrón específico y recurrente de aberraciones presente en un alto porcentaje de casos (78%). Estas aberraciones genómicas coincidían con las denominadas Ar-CNV que describimos como un patrón de aCGH específico tanto en muestras normales como en diferentes tipos tumorales (leucemias, linfomas, cáncer de mama, etc.), independientemente de la plataforma de array (array de oligos o de BACs) y del laboratorio donde se realizó la hibridación (Blesa *et al.*, en preparación). Para neutralizar el efecto de los artefactos y poder analizar los casos, desarrollamos un protocolo de estandarización similar al desarrollado por Kirchhoff y cols. en un estudio de cCGH diseñado para evitar un problema similar (ver Material y Métodos 5.1.3) (Kirchhoff *et al.*, 1998). La Figura 11 de esta tesis sirve de ejemplo de la estandarización desarrollada durante el análisis de los casos con artefactos. La utilidad de este procedimiento quedó de manifiesto al realizar experimentos FISH en algunas de las regiones afectadas por los artefactos (Material y Métodos 3.4.1), los cuales mostraron un importante crecimiento en el número de aberraciones genómicas confirmadas (de un 9% a un 82% antes y después de la estandarización, respectivamente).

2.2. CARACTERIZACIÓN GENÓMICA POR ARRAY DE CGH DE LAS CLASES DE CÁNCER DE MAMA: *BRCA1*, *BRCA2*, BRCAX Y ESPORÁDICO

2.2.1. Inestabilidad genómica en las clases de cáncer de mama

Se determinó el grado de inestabilidad genómica de acuerdo al número medio de cambios genómicos (ganancias, pérdidas y totales) presentes en un tumor. Los tumores asociados a mutación de *BRCA1* presentaron la mayor inestabilidad genómica con un total de 28'0±2'9 aberraciones totales por tumor, los asociados a mutación de *BRCA2* presentaron 19'8±2'3, mientras que los BRCAX mostraron 15'3±1'9 y los tumores esporádicos un total de 18'7±1'9 (Figura 18). Estas diferencias fueron estadísticamente significativas siempre que se comparaban el número de aberraciones de los tumores *BRCA1* con cualquiera de las otras clases tumorales (Figura 18).

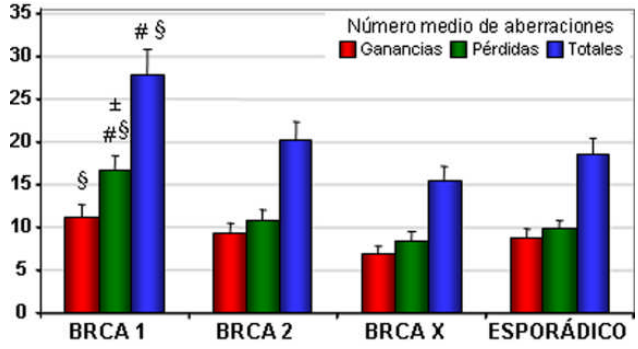


Figura 18. Número medio de aberraciones genómicas por clase de cáncer de mama. Se muestran las diferencias significativas (p-valor<0'05 en el Test de U-Mann Whitney) cuando se comparan tumores *BRCA1* frente a *BRCA2* (±), *BRCA1* frente a BRCAX (§), o *BRCA1* frente a tumores esporádicos (#).

2.2.2. Frecuencias de cambios genómicos en las clases de cáncer de mama

La frecuencia y distribución de las aberraciones genómicas (ganancias o pérdidas) en cada una de las clases fueron ligeramente diferentes (Figura 19). Cuatro regiones genómicas se alteraban con una frecuencia mayor del 40% en todas las clases tumorales: ganancias de 1q y 16p13.3, y pérdidas de 8ptel-p12 y 16q (flechas en Figura 19). En la búsqueda de aberraciones asociadas con alguna de las clases de cáncer de mama, encontramos algunas diferencias estadísticamente significativas en las comparaciones entre dos clases (Tabla 6). Sin embargo, no se registró ninguna aberración exclusivamente asociada a una clase tumoral. Las diferencias en las frecuencias de alteración del cromosoma X no fueron estudiadas ya que la serie de muestras esporádicas se había hibridado con ADN del sexo contrario.

Tabla 6. Aberraciones estadísticamente significativas, sus frecuencias entre las distintas clases y las frecuencias de amplificaciones de alto nivel.

	Regiones cromosómicas	Frecuencias de aberración				Valor de P en el test exacto de Fisher					
		BRCA1	BRCA2	BRCAX	SPORADIC	B1vsB2	B1vsBX	B1vsE	B2vsBX	B2vsE	BXvsE
Ganancia	8q22.1	**58**	**79**	32	**74**				**0'023**		
	8q23.1-8q23.3	**74**	**83**	35	**74**				**0'010**		
	8q23.3-8q24.13	**79**	**83**	35	**74**				**0'010**		
	8q24.13-8qtel	**84**	**83**	39	**74**		0'077		**0'025**		
	11q14.1	47	0	10	10	**0'005**					
Pérdida	4q23	32	12	6	53						**0'020**
	4q24	37	12	6	**58**						**0'005**
	4q25	47	12	6	**58**		0'074				**0'005**
	4q26-4q28.1	42	12	6	**58**						**0'005**
	4q28.2	42	16	9	**58**						**0'031**
	4q28.3	42	16	10	**63**						**0'022**
	4q31.1	42	8	13	**63**					**0'009**	**0'022**
	4q31.21	37	8	16	**63**					**0'009**	
	4q32.1-4q32.2	**47**	12	23	63					**0'044**	
	4q32.3	**52**	12	23	63					**0'044**	
	4q33-4q34.1	**58**	12	26	63					**0'044**	
	4q34.2-4q35.1	**58**	12	23	63					**0'044**	
	4q35.1-4q35.2	**52**	12	26	63					**0'044**	
	13q12.3	47	**50**	16	**68**						**0'013**
	13q13.1-13q13.3	**53**	**50**	16	**68**						**0'013**
	13q13.3-13q14.11	**58**	**54**	16	**68**						**0'013**
	13q14.11-13q14.3	**63**	**54**	16	**68**		0'097				**0'013**
	13q14.3-13q21.33	**68**	**54**	16	**58**		**0'019**				
	13q22.1-13q22.2	**68**	42	16	**58**		**0'019**				
	13q22.2-13q22.3	**68**	42	19	**58**		**0'048**				
	13q31.1	**63**	42	19	**53**		**0'026**				
	21q21.3	11	0	3	42					0'025	0'046
Amplificaciones de alto nivel											
	8p12-8p11.23	11	8	6	**16**						
	8p11.22-8pcen	11	4	3	**16**						
	8q22.1-8q22.3	5	**17**	10	**16**						
	8q23.1-8q23.3	11	**21**	10	**21**						
	8q24.11	**32**	**37**	**16**	**21**						
	8q24.12-8q24.21	**37**	**37**	**16**	**21**						
	8q24.21-8q24.22	**37**	**29**	13	**26**						
	8q24.23-8qtel	**21**	**25**	10	**26**						
	10ptel-10p15.3	0	0	0	**16**						
	10p15.2	11	0	0	**16**						
	10p15.1	0	0	0	**16**						
	10p14	5	0	0	**16**						
	11q13.3-11q13.4	11	12	**16**	0						
	12p13.32-12p13.31	**16**	0	0	5						
	13q34-13qtel	**16**	4	0	11						

Frecuencias ≥50% (para ganancia/pérdida) y ≥15% (para amplificaciones) se muestran en negrita Abreviaturas: B1, tumores *BRCA1*; B2, tumores *BRCA2*; y BX, tumores BRCAX. Se muestran los valores de *P* inferiores a 0'1 y en negrita aquellos menores a 0'05. Se exponen las amplificaciones de alto nivel con una frecuencia mayor a 15% en alguna de las clases.

En cada una de las clases tumorales, a parte de las aberraciones comunes, existieron ciertas regiones con una frecuencia de alteración superior al 50% (Figura 19). Por ejemplo, pérdidas de 4q32.3-qtel, 5q, 13q y 18q fueron más frecuentes en tumores asociados a *BRCA1*. Los tumores asociados a *BRCA2* presentaron ganancias recurrentes en 8q12.3-qtel y pérdidas de 11q23.1-qtel, 13q12.3-q21.33 y del cromosoma 22. Los cánceres esporádicos tenían frecuentemente ganancias de 8q13.1-qtel y pérdidas de 4q24-qtel y 13cen-q31.3. Por último, los tumores BRCAX no mostraron ninguna aberración recurrente aparte de las comunes.

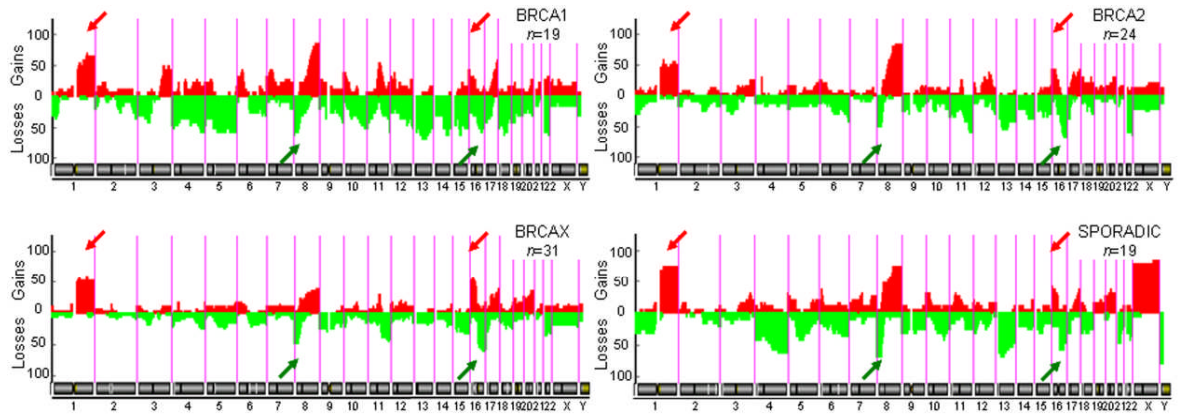


Figura 19. Perfil de aberraciones genómicas de los tumores *BRCA1*, *BRCA2*, BRCAX y esporádicos. En rojo, las frecuencias de ganancia; verde, las frecuencias de pérdida. Las flechas en los cromosomas 1, 8 y 16 señalan regiones comúnmente alteradas en más del 40% en todas las clases de tumor.

En cuanto a las amplificaciones de alto nivel, 8q24.22 y 8q24.12-q24.21 fueron las dos únicas regiones con una frecuencia mayor del 15% en todas las clases de cáncer de mama. No se registraron diferencias significativas en cuanto a la frecuencia de estas aberraciones, si bien existían leves diferencias en cuanto a la frecuencia y localización de las mismas (Tabla 6). Quisimos comprobar en detalle algunas regiones concretas de amplificación conocidas por contener oncogenes conocidos, tales como 8q24.21 (*MYC*) o 17q12 (*ERBB2*). La amplificación de *MYC* se registró en todos los grupos (37% en tumores *BRCA1*, 33% *BRCA2*, 26% esporádico y 13% en BRCAX). Por su parte, la amplificación de *ERBB2* se daba en un 12’9% de BRCAX y en un 5’3% de las muestras esporádicas estudiadas, mientras que ninguno de los tumores *BRCA1/2* presentaba amplificación de esta región cromosómica.

2.3. VALIDACIÓN DE UN CLASIFICADOR *BRCA*

Durante el inicio del presente estudio, el primer análisis del cáncer de mama familiar *BRCA1* y *BRCA2* usando una plataforma de aCGH fue publicado (Jonsson *et al.*, 2005). Los autores confirmaron la mayor inestabilidad genómica de los tumores *BRCA1* y las pérdidas específicas de las regiones 4p, 4q y 5q; así como la mayor frecuencia de ganancias en 17q24 de los tumores *BRCA2*, características éstas descritas previamente (ver Tabla 1). Los autores también propusieron una serie de regiones cromosómicas capaces de discriminar la clase de tumor *BRCA1* ó *BRCA2* en función de las aberraciones genómicas presentes en las susodichas regiones. Cabe destacar que la plataforma de aCGH utilizada

por Jönsson y cols. fue la misma que la que nosotros empleamos en nuestros estudios (Greshock *et al.*, 2004).

Con el objetivo de validar el "clasificador" propuesto por Jönsson y cols., realizamos un agrupamiento jerárquico no supervisado utilizando nuestra serie de muestras y las mismas regiones discriminativas que los autores habían descrito. Para ello, solo incluimos los tumores esporádicos y los procedentes de portadores de mutación en *BRCA1* ó *BRCA2*, puesto que éstas fueron las clases utilizadas para hallar las regiones discriminativas. Como resultado, obtuvimos dos subgrupos: uno de ellos principalmente formado por tumores *BRCA1* (rama naranja) y el otro constituido en su mayoría por tumores *BRCA2* y esporádicos (rama verde) (Figura 20A). Tal y como Jönsson describió, la rama *BRCA1* se caracterizaba por las ganancias en 3q24-q25 y las pérdidas en 4p, 4q y 5q, y la rama *BRCA2* por una mayor frecuencia de ganancias en 17q24. Sin embargo, encontramos varios tumores *BRCA2* (4 casos) y esporádicos (6) erróneamente clasificados como *BRCA1* y tumores *BRCA1* (7) localizados en la rama *BRCA2*. Jönsson y cols. también encontraron muestras mal clasificadas en su agrupamiento y sugirieron como razones el estado del receptor de estrógenos (RE) y el grado histológico. Al contrastar estos parámetros en nuestra serie, vimos que más de la mitad de las muestras erróneamente clasificadas en la rama *BRCA1* (2 *BRCA2* y 5 casos esporádicos) eran RE-negativo, y que 6 de las 7 muestras *BRCA1* clasificadas como *BRCA2* presentaban RE-positivo (Figura 20A). Esto podría indicar que las regiones descritas estaban discriminando tumores en función del estado del RE en lugar de mutación en los genes *BRCA*. Por su parte, el grado histológico estaba más repartido en ambas ramas (datos no mostrados).

Al mirar en detalle la serie de Jönsson y cols., nos dimos cuenta de que todas las muestras *BRCA1* eran RE-negativas y todas las muestras *BRCA2* eran RE-positivas, mientras que los casos esporádicos presentaban ambos estados de RE. En nuestro estudio, todas nuestras clases de tumores tenían ambos estados de RE, por lo que decidimos quitar del agrupamiento aquellas muestras que no cumplían los atributos de la serie de Jönsson: los casos RE-positivos *BRCA1* y los RE-negativos *BRCA2*. En esta ocasión, obtuvimos un agrupamiento distinto en el que volvimos a obtener dos grupos: uno de ellos (rama naranja) compuesto principalmente por tumores RE-negativos (17 casos RE-negativos y 2 RE-positivos) y un segundo grupo (rama verde) formado por tumores RE-positivos (22 casos RE-positivos y 3 RE-negativos) (Figura 20B). El número de muestras BRCA que se

clasificaban erróneamente se redujo de 11 muestras (7 *BRCA1* y 4 *BRCA2*) (Figura 20A) del anterior agrupamiento a solo 3 muestras (1 *BRCA1* y 2 *BRCA2*) (Figura 20B). Esta clasificación se asemejaba a los resultados obtenidos por Jönsson, pero a la vez, señalaba los problemas en el diseño de estos estudios comparativos de clases tumorales y, especialmente, la importancia que el estado del RE podría tener en el desarrollo tumoral.

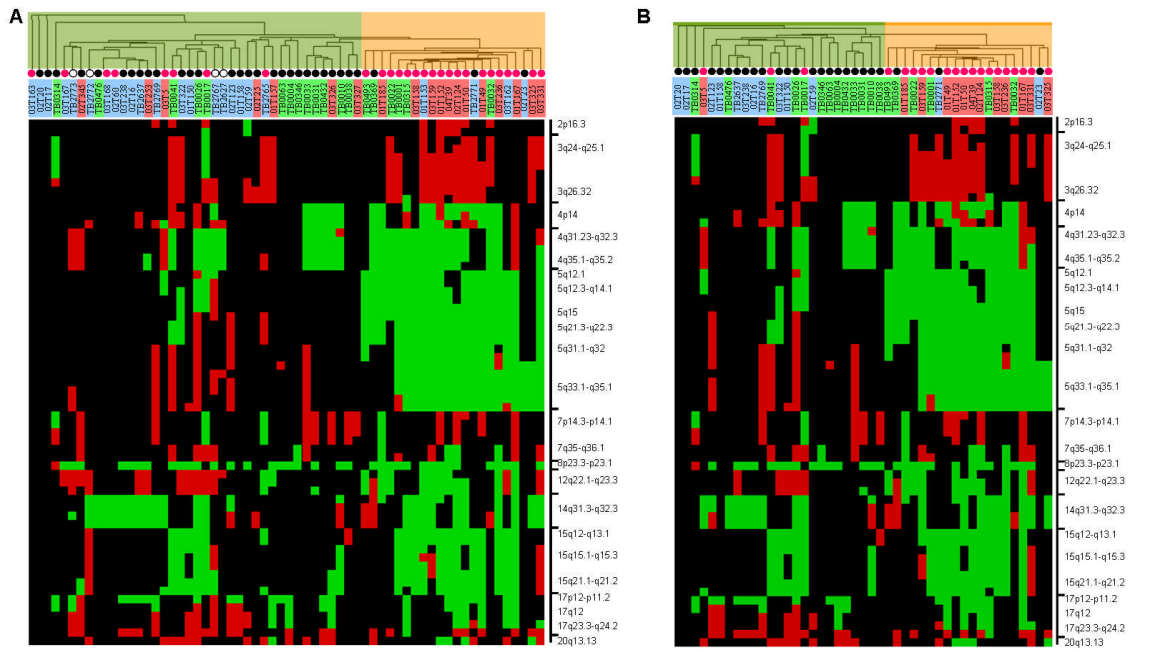


Figura 20. Clasificador propuesto por Jönsson usando nuestra serie de casos: A, muestras BRCA1 (rojo), BRCA2 (azul) y esporádicos (verde). B, muestras RE-negativo BRCA1, RE-positivo BRCA2 y esporádicos. Las regiones cromosómicas que componen el clasificador se muestran en la derecha del agrupamiento y muestran ganancias (en rojo) y pérdidas (en verde) por cada caso. El estado del RE se muestran con círculos negro (positivo), rojo (negativo), blanco (datos no disponible).

2.4. EL PATRÓN GENÓMICO EN FUNCIÓN DEL ESTADO DEL RECEPTOR DE ESTRÓGENOS

Para investigar el papel del RE, dividimos todos los casos de acuerdo al estado del RE, contabilizamos el número medio de aberraciones genómicas y diseñamos el patrón de aberraciones genómicas en cada grupo. La mayor inestabilidad genómica de los tumores RE-negativos fue patente, mostrando diferencias significativas (P<0’05) en el número medio de cambios (total, ganancias y pérdidas) cuando se comparaba con los tumores RE-positivos (Figura 21A). Estas diferencias también se mostraban cuando se subdividían los grupos en función de la clase de tumor y del estado de RE (Figura 21B).

Los pérfiles de aberraciones genómicas de los grupos de tumor en función del RE se muestran en la Figura 22A. Ambos grupos de tumores tenían ganancias comunes de 1q y

8q22.1-qtel y pérdidas comunes de 8ptel-p12 y 16q, los cuales eran similares a los descritos en todas las clases tumorales. Sin embargo, un conjunto de aberraciones genómicas, tales como -3p25.3-p21.3, -3p21.1-p14.2, +3q25.1-q26.1, 4q31.2-qtel, -5q, +10p14-p12.3, -12q14-q15 y -12q23.1-q23.31, presentaron de manera estadísticamente significativa una mayor frecuencia en los tumores RE-negativos (Figura 22B). Cabría destacar que la región 6q25.1 (locus del gen *ESR*) registró una mayor frecuencia de pérdida en los tumores RE-positivos que en los tumores RE-negativos. Finalmente, los patrones de aberraciones genómicas también mostraron su heterogeneidad cuando cada clase de tumor se subdividió según su estado de RE (Figura S 1).

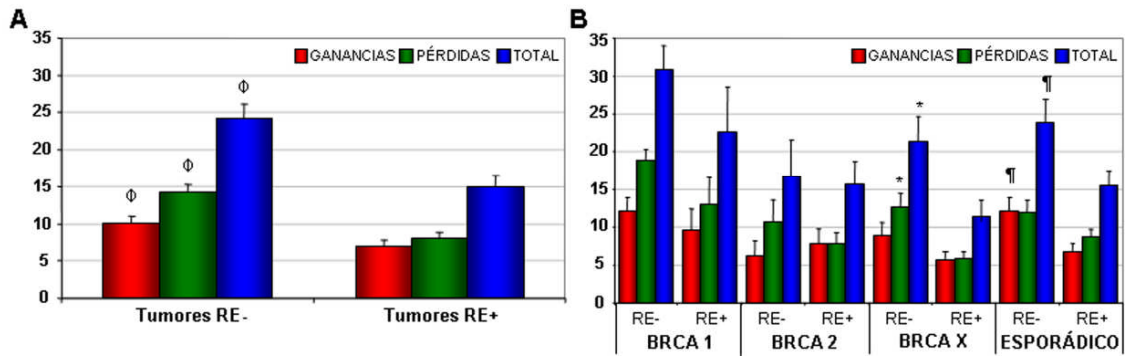


Figura 21. Número medio de cambios genómicos por tumor de acuerdo al estado del receptor de estrógenos. A, sin subclasificar por mutación BRCA. B, subclasificando según mutación BRCA. Las diferencias estadísticas significativas en el análisis no paramétrico de U de Mann-Whitney se muestran como (Φ) al comparar tumores RE(-) y RE(+), (*) al comparar tumores BRCAX RE(-) y RE(+) y (¶) al comparar tumores esporádicos RE(-) y RE(+).

En lo que respecta a las amplificaciones de alto nivel, observamos que los tumores RE-negativos tenían una tendencia a desarrollar un mayor número de amplificaciones que los tumores RE-positivos y además presentaron ligeras diferencias en cuanto a las regiones afectadas con esta aberración genómica. Por ejemplo, la amplifcación de 8q24.21 (locus del gen *c-MYC*) se registró en un 32% de los tumores RE-negativos y en un 20% de los tumores RE-positivos. Por su parte, 17q12-q21 (*ERBB2*) solo se encontró amplificado en tumores RE-negativos (5%), mientras que la amp20q13.12-q13.33 solo se registró en los tumores RE-positivos (8%). Sin embargo, de todas las amplificaciones de alto nivel, la única que presentó diferencias estadísticamente significativas fue la amp13q34, exclusiva de tumores RE-negativos (16%).

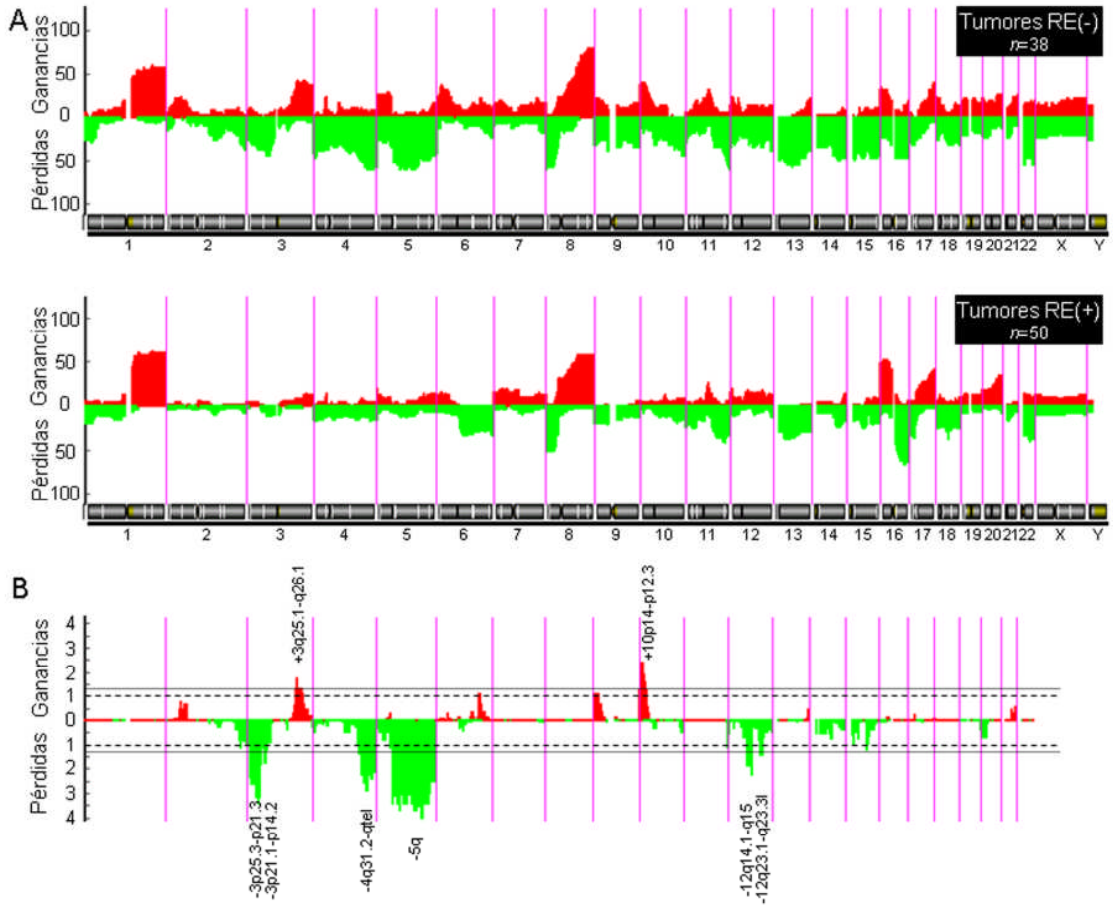


Figura 22. Patrón de aberraciones genómicas en los grupos de tumor clasificados por el estado de RE. A, patrones en tumores RE(-) y RE(+) donde se muestran las frecuencias de ganancias (rojo) y pérdidas (verde). B, representación del valor negativo del logaritmo del valor de P ajustado, obtenido de la prueba exacta de Fisher al comparar las frecuencias de ganancias (rojo) y pérdidas (verdes) entre los tumores RE(-) y RE(+). Las líneas intermitentes muestran el valor de P=0'1 (-log(P ajustada)=1), mientras que las líneas continuas muestran el valor de P=0'05 (-log(P ajustada)=1.3). Las aberraciones cuya diferencia en frecuencias es estadísticamente significativa se muestran representadas.

En resumen, con este estudio pudimos caracterizar el patrón de aberraciones genómicas de las diferentes clases de cáncer de mama: *BRCA1*, *BRCA2*, BRCAX y esporádico. Fuimos capaces de confirmar algunas de las aberraciones frecuentes dentro de cada una de estas clases y validamos un clasificador de aCGH. Gracias a ello, se vio que el cáncer de mama familiar presenta también una heterogeneidad que responde al estado del RE, por lo que nuestros resultados sugieren que este marcador habría de tenerse en cuenta a la hora de diseñar estudios comparativos de casos esporádicos y familiares.

RESULTADOS PARTE III

Los patrones de aberraciones genómicas en el cáncer de mama familiar difieren en función del subtipo inmunohistoquímico

En el cáncer de mama esporádico se han descrito cinco subtipos moleculares mediante análisis de la expresión génica: basal, ERBB2, los luminales A y B y el subtipo similar a tejido normal. Se ha visto que estos subtipos de cáncer de mama esporádico presentan diferentes patrones de aberraciones genómicas. Dado que nuestro grupo describió recientemente estos subtipos moleculares en una serie de 50 tumores BRCAX usando ensayos de inmunohistoquímica, decidimos extender el estudio a todas las clases de cáncer de mama familiar incluyendo un total de 62 tumores (18 BRCA1*, 16* BRCA2 *y 28 BRCAX) mediante el empleo del mismo panel de marcadores inmunohistoquímicos y del grado histológico. De este modo, obtuvimos una clasificación similar si bien los tumores* BRCA1 *se asociaron al fenotipo basal, mientras que los tumores BRCAX lo hacían al subtipo luminal A. Al combinar estos resultados con los datos cosechados a partir de nuestro estudio con aCGH de 1Mb, describimos diferentes patrones de aberraciones genómicas asociados a cada subtipo inmunohistoquímico. Los tumores basales mostraron un mayor número de ganancias y pérdidas genómicas, mientras que los tumores luminales B desarrollaron un mayor número de amplificaciones de alto nivel. Nuestros resultados se asemejan a los obtenidos en los estudios con cáncer de mama esporádico, señalando la existencia de diferentes rutas genéticas de evolución tumoral comunes al cáncer de mama esporádico y al familiar.*

3. SEGUNDO ESTUDIO DE ARRAY CGH: LOS PATRONES DE ABERRACIONES GENÓMICAS EN CÁNCER DE MAMA FAMILIAR DIFIEREN EN FUNCION DEL SUBTIPO INMUNOHISTOQUÍMICO

Tras haber observado la heterogeneidad en función del estado del RE en cada una de las clases de cáncer de mama familiar, la siguiente pregunta que nos surgió durante esta tesis fue: ¿somos capaces de reproducir la misma heterogeneidad en el cáncer de mama familiar como la que presenta el cáncer de mama esporádico? Tal y como comentamos en la Introducción, el cáncer de mama esporádico se divide en al menos cinco subtipos a tenor de los resultados de varios estudios de expresión (Hu *et al.*, 2006; Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003; Sotiriou *et al.*, 2003). Durante el año 2006, dos estudios asociaron diferentes patrones de aberraciones genómicas a cada uno de los subtipos moleculares de los tumores esporádicos (Bergamaschi *et al.*, 2006; Chin *et al.*, 2006). Por lo tanto, nuestros objetivos con el presente estudio fueron: 1) ¿puede el cáncer de mama familiar ser clasificado en diferentes subtipos?, 2) si es así, ¿cada subtipo tiene un patrón de aberraciones característico?, y 3) ¿qué interpretación se puede dar a estos resultados?.

3.1. EL CÁNCER DE MAMA FAMILIAR TAMBIÉN SE SUBDIVIDE EN DIFERENTES SUBTIPOS MOLECULARES

Dado que nuestra serie procedía de tumores embebidos en parafina, en los que cualquier estudio de arrays de expresión tiene poca fiabilidad, enfocamos la búsqueda de los diferentes subtipos moleculares en función de los datos cosechados con nuestros análisis de expresión proteica (IHQ). En este sentido, un reciente estudio desarrollado por nuestro grupo reprodujo la heterogeneidad registrada en el cáncer de mama esporádico en una serie de 50 tumores BRCAX. En el susodicho análisis, se utilizaron 25 marcadores de IHQ y el grado histológico para agrupar las diferentes muestras (Honrado *et al.*, 2007). Por lo tanto, para abordar el presente estudio, solo se incluyeron aquellas muestras que habían sido analizadas tanto por aCGH como por IHQ obteniendo una serie de 62 muestras (18 *BRCA1*, 16 *BRCA2* y 28 BRCAX).

Realizamos un agrupamiento jerárquico no supervisado de las 62 muestras con los 25 marcadores de IHQ (ver Tabla 3) y el grado histológico. Las muestras se clasificaron en dos grupos principales, de nuevo asociados con el estado del RE. La rama de los tumores RE-negativos (Figura 23, izquierda) se compuso de 20 casos de grado 2 y 3 (cuadrados marrón y rojo, respectivamente), también caracterizados por la negatividad para receptores hormonales y la sobreexpresión de proteinas implicadas en la progresión del ciclo celular y proliferación celular. Por su parte, la rama de tumores RE-positivos incluyó 42 casos mayoritariamente de grado 1 (cuadrados verdes) ó 2, positividad para receptores hormonales y expresión de proteinas relacionadas con la inhibición de los complejos ciclina-CDQ y proteinas luminales (como citoqueratina 8, CK8) (Figura 23, derecha).

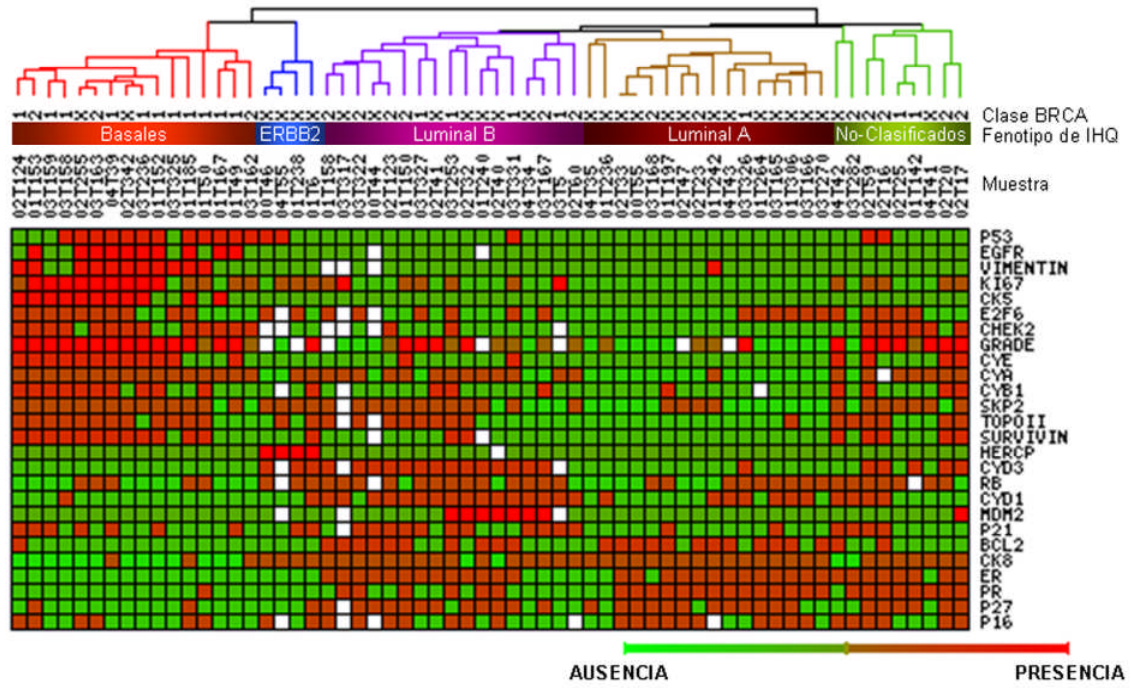


Figura 23. Agrupamiento jerárquico no supervisado de las 62 muestras de cáncer de mama familiar usando 25 marcadores de IHQ y grado histológico. Las ramas están coloreadas y definen cada uno de los subtipos IHQ que aparecen reflejados como rectángulos coloreados con su nombre respectivo. Los cuadrados blancos corresponden a datos no disponibles. El porcentaje de células positivas para cada marcador IHQ se representa como un rango de color desde el más verde (el menor porcentaje) hasta el más rojo (el mayor porcentaje). Los colores intermedios representan porcentajes entre el menor y el mayor.

La rama de tumores RE-negativos se podía subdividir en dos subgrupos: uno caracterizado por la sobreexpresión de ERBB2 (4 tumores) y un segundo subgrupo (de 16 tumores) cuya peculiaridad radicaba en la sobreexpresión de marcadores basales tales como citoqueratina 5 (CK5), vimentina, survivina y el receptor del factor de crecimiento epidérmico (EGFR). El subgrupo asociado a la sobreexpresión de ERBB2 se compuso

exclusivamente de tumores BRCAX, mientras que el subgrupo basal incluyó mayoritariamente tumores *BRCA1* (11 de 16 casos).

Tabla 7. Distribución de los tumores familiares en los diferentes subtipos IHQ.

	Basal	ERBB2	Luminal B	Luminal A	No-Clasificado
BRCA1 (n=18)	**11 (61'1%)†**	0	4 (22'2%)	1 (5'6%)	2 (11'1%)
BRCA2 (n=16)	3 (18'8%)	0	**6 (37'5%)**	2 (12'5%)	**5 (31'2%)**
BRCAX (*n*=28)	2 (7'1%)	4 (14'3%)	7 (25.0%)	**13 (46'5%)‡**	2 (7'1%)
Total (*n*=62)	16 (25'8%)	4 (6'5%)	17 (27'4%)	16 (25'8%)	9 (14'5%)

Los valores en negrita indican el fenotipo IHQ que más representado está dentro de cada clase de tumor BRCA. Se señalan las diferencias significativas con la prueba exacta de Fisher (P≤0'05) en la distribución basal de *BRCA1* frente a *BRCA2* y de *BRCA1* frente a BRCAX (†), así como la distribución luminal A de BRCAX frente a *BRCA1* y BRCAX frente a *BRCA2* (‡).

Por otro lado, tres subgrupos podrían definirse dentro de la rama de tumores RE-positivos. Uno de ellos (Figura 23, rama marrón) se compuso de 16 tumores mayoritariamente de grado 1, que sobre-expresaban receptores hormonales, CK8, BCL2 y proteinas represoras del ciclo celular (p27 y p16). La mayoría de estos tumores fueron BRCAX (13 de 16). Un segundo subgrupo estaba formado por un total de 17 muestras (Figura 23, rama violeta), las cuales se caracterizaban por un mayor grado histológico que el anterior subgrupo, así como por una menor o incluso ausencia de expresión de receptores hormonales comparado con los otros subgrupos de tumores RE-positivos. También presentaron una sobreexpresión de otras proteinas tales como ciclina A (relacionada con la progresión del ciclo celular) y TOPOII (relacionada con el crecimiento celular). En lo que respecta a la composición de este subgrupo, se registró una mezcla de las tres clases familiares de cáncer de mama. A estos dos subgrupos, los denominamos luminal A y luminal B respectivamente de acuerdo a ciertos parámetros (tales como el grado histológico y la expresión diferencial de TOPOII) usados previamente para discriminar entre estos dos subtipos moleculares (Sorlie *et al.*, 2001; Sotiriou *et al.*, 2003). Por último, un subgrupo de 9 tumores (Figura 23, rama verde) terminaba de conformar la rama de los tumores RE-positivos y se definía por la presencia de marcadores luminales (RE+, RP+, CK8+), un alto grado histológico y la sobreexpresión de ciertos marcadores como CHEK2 y survivina. En lo que respecta a su composición, este grupo de tumores se constituía principalmente de tumores *BRCA2* (5 de las 9 muestras). Dado que los rasgos de este subgrupo no coincidían con los previamente establecidos para otros subtipos

moleculares, lo denominamos subgrupo "no-clasificado" pero podría ser catalogado como luminal C ó luminal 3.

Un resumen de la distribución de las diferentes clases de cáncer de mama familiar se detalla en la Tabla 7. La mayoría de los tumores *BRCA1* mostraron un fenotipo basal, mientras que los tumores *BRCA2* se localizaban principalmente en los subtipos luminal B ó no-clasificado. El subtipo ERBB2 solo se compuso de tumores BRCAX, aunque la mayoría de los tumores correspondientes a esta clase desarrollaron un fenotipo luminal A. Las características IHQ de los diferentes subtipos y las comparaciones estadísticas se registran en la Tabla 8, mostrando que, como cabía esperar, cada uno de los subtipos presentaba diferencias significativas en aquellos marcadores que los caracterizaban. Lo que es digno de resaltar tras este agrupamiento es que cada una de las clases de cáncer de mama familiar no representa una clase totalmente homogénea (como se venía suponiendo especialmente en tumores *BRCA1/2*), sino que existe una heterogeneidad que se asemeja a la registrada en el cáncer de mama esporádico.

Tabla 8. Marcadores IHQ y comparaciones estadísticas entre los diferentes subtipos de IHQ

	Basal	ERBB2	Luminal B	Luminal A	No-Clasificado	P1	P2	P3	P4	P5
	(*n*=16)	(*n*=4)	(*n*=17)	(*n*=16)	(*n*=9)					
HERCP(+3)										
1-2 +	16 (100'0)	0	16 (100'0)	16 (100'0)	9 (100'0)	NS	0'000	NS	NS	NS
3 +	0	4 (100'0)	0	0	0					
RE										
Negativo	16 (100'0)	4 (100'0)	4 (23'5)	3 (18'8)	1 (11'1)	0'000	0'037	0'047	0'019	0'033
Positivo	0	0	13 (76'5)	13 (81'3)	8 (88'9)					
RP										
Negativo	15 (93'8)	4 (100'0)	11 (64'7)	2 (12'5)	1 (11'1)	0'000	NS	NS	0'000	0'009
Positivo	1 (6'2)	0	6 (35'3)	14 (87'5)	8 (88'9)					
P53										
Negativo	4 (25'0)	2 (50'0)	16 (94'1)	16 (100'0)	7 (77'8)	0'000	NS	0'025	0'003	NS
Positivo	12 (75'0)	2 (50'0)	1 (5'9)	0	2 (22'2)					
BCL2										
Negativo	15 (93'8)	4 (100'0)	7 (41'2)	4 (25'0)	7 (77'8)	0'001	NS	0'086	0'002	NS
Positivo	1 (6'3)	0	10 (58'8)	12 (75'0)	2 (22'2)					
Ki-67										
0-4%	2 (12'5)	1 (25'0)	10 (58'8)	16 (100'0)	4 (44'4)	0'000*	0'105*	NS*	0'000*	0'102*
5-24%	5 (31'3)	3 (75'0)	5 (29'4)	0	5 (55'6)					
25-100%	9 (56'3)	0	2 (11'8)	0	0					
EGFR										
Negativo	6 (37'5)	4 (100'0)	15 (100'0)	16 (100'0)	9 (100'0)	0'000	NS	0'054	0'050	NS
Positivo	10 (62'5)	0	0	0	0					
CK5										
Negativo	5 (31'3)	4 (100'0)	17 (100'0)	16 (100'0)	9 (100'0)	0'000	NS	0'026	0'052	NS
Positivo	11 (68'8)	0	0	0	0					
Vimentina										
Negativo	5 (31'3)	4 (100'0)	14 (100'0)	15 (93'8)	9 (100'0)	0'000	NS	0'052	NS	NS
Positivo	11 (68'8)	0	0	1 (6'3)	0					
Grado										
1	0	1 (50'0)	4 (28'6)	10 (71'4)	1 (11'1)	0'001*	NS*	0'066*	0'000*	NS*
2	2 (12'5)	0	6 (42'9)	3 (21'4)	1 (11'1)					
3	14 (87'5)	1 (50'0)	4 (28'6)	1 (7'1)	7 (77'8)					
CK8										
Negativo	13 (81'3)	0	2 (11'8)	0	2 (22'2)	0'000	NS	NS	0'003	NS
Positivo	3 (18'8)	4 (100'0)	15 (88'2)	16 (100'0)	7 (77'8)					

Ciclina D1										
Negativo	15 (93'8)	3 (75'0)	1 (5'9)	9 (56'3)	5 (55'6)	0'000	NS	0'000	NS	NS
Positivo	1 (6'3)	1 (25'0)	16 (94'1)	7 (43'8)	4 (44'4)					
Ciclina D3										
Negativo	16 (100'0)	0	1 (6'7)	15 (93'8)	4 (44'4)	0'000	0'054	0'000	0'002	NS
Positivo	0	3 (100'0)	14 (93'3)	1 (6'3)	5 (55'6)					
Ciclina E										
Negativo	4 (25'0)	4 (100'0)	15 (88'2)	16 (100'0)	5 (55'6)	0'000	NS	NS	0'003	NS
Positivo	12 (75'0)	0	2 (11'8)	0	4 (44'4)					
Ciclina A										
Negativo	0	2 (50'0)	4 (23'5)	15 (93'8)	1 (12'5)	0'000	NS	NS	0'000	NS
Positivo	16 (100'0)	2 (50'0)	13 (76'5)	1 (6'3)	7 (87'5)					
Ciclina B1										
Negativo	6 (37'5)	2 (66'7)	13 (81'3)	14 (93'3)	5 (55'6)	0'004	NS	NS	0'023	NS
Positivo	10 (62'5)	1 (33'3)	3 (18'8)	1 (6'7)	4 (44'4)					
P27										
Negativo	14 (87'5)	3 (75'0)	4 (25'0)	4 (25'0)	3 (33'3)	0'000	NS	0'079	0'079	NS
Positivo	2 (12'5)	1 (25'0)	12 (75'0)	12 (75'0)	6 (66'7)					
SKP2										
Negativo	2 (12'5)	0	4 (25'0)	12 (75'0)	2 (22'2)	0'063	NS	NS	0'000	NS
Positivo	14 (87'5)	4 (100'0)	12 (75'0)	4 (25'0)	7 (77'8)					
P16										
Negativo	10 (62'5)	1 (33'3)	7 (50'0)	5 (33'3)	3 (33'3)	NS	NS	NS	NS	NS
Positivo	6 (37'5)	2 (66'7)	7 (50'0)	10 (66'7)	6 (66'7)					
P21										
Negativo	9 (56'3)	2 (50'0)	5 (31'3)	14 (87'5)	5 (55'6)	NS	NS	0'019	0'007	NS
Positivo	7 (43'8)	2 (50'0)	11 (68'8)	2 (12'5)	4 (44'4)					
RB										
Negativo	11 (68'8)	0	3 (18'8)	7 (43'8)	1 (12'5)	0'005	NS	NS	NS	NS
Positivo	5 (31'3)	3 (100'0)	13 (81'3)	9 (56'3)	7 (87'5)					
MDM2										
Negativo	16 (100'0)	3 (100'0)	8 (53'3)	16 (100'0)	8 (88'9)	0'093	NS	0'000	0'093	NS
Positivo	0	0	7 (46'7)	0	1 (11'1)					
E2F6										
Negativo	6 (37'5)	1 (33'3)	9 (60'0)	10 (62'5)	4 (44'4)	NS	NS	NS	NS	NS
Positivo	10 (62'5)	2 (66'7)	6 (40'0)	6 (37'5)	5 (55'6)					
TOPOII										
Negativo	4 (25'0)	2 (50'0)	7 (46'7)	15 (93'8)	3 (33'3)	0'019	NS	NS	0'000	NS
Positivo	12 (75'0)	2 (50'0)	8 (53'3)	1 (6'3)	6 (66'7)					
CHEK2										
Negativo	3 (18'8)	2 (100'0)	11 (84'6)	16 (100'0)	3 (33'3)	0'000	NS	0'101	0'000	0'066
Positivo	13 (81'3)	0	2 (15'4)	0	6 (66'7)					
Survivina										
Negativo	4 (25'0)	3 (75'0)	13 (86'7)	16 (100'0)	4 (44'4)	0'000	NS	0'067	0'001	NS
Positivo	12 (75'0)	1 (25'0)	2 (13'3)	0	5 (55'6)					

Se muestran los valores de *P* de la prueba exacta de Fisher cuando se compara un subtipo de IHQ frente al resto: P1, tumores basales frente al resto; P2, tumores ERBB2 frente al resto; P3, tumores luminal B frente al resto; P4, tumores luminal A frente al resto; P5, tumores no-clasificados frente al resto. (*) La prueba chi-cuadrado se usó para Ki-67 y el grado.
Los valores de P≤0'10 se representan. Solo se consideran significativos aquellos P≤0'05. NS, significa no significativo.

3.2. CARACTERIZACIÓN GENÓMICA DE LOS SUBTIPOS DE CÁNCER DE MAMA FAMILIAR

Una vez que los tumores familiares fueron clasificados en función de su patrón de IHQ, recopilamos los datos de aCGH de estas muestras y contabilizamos el número medio y el perfil de aberraciones genómicas, asi como el número y localización de amplificaciones de alto nivel en cada uno de los subtipos de IHQ definidos.

Los tumores de fenotipo basal presentaron un mayor número de aberraciones genómicas (30'75±3'0), mientras que los tumores con un subtipo luminal A fueron los que menor inestabilidad genómica mostraron (10'87±1'9). Los tumores luminal B, ERBB2 y no-clasificados tuvieron un número medio de 20'00±2'7, 14'75±6'2 y 14'44±3'7

aberraciones genómicas por caso, respectivamente (Figura 24). Cualquier comparación en el número medio de desequilibrios genómicos entre el subtipo basal y los restantes presentaron diferencias estadísticamente significativas (p<0'05, prueba U de Mann-Whitney).

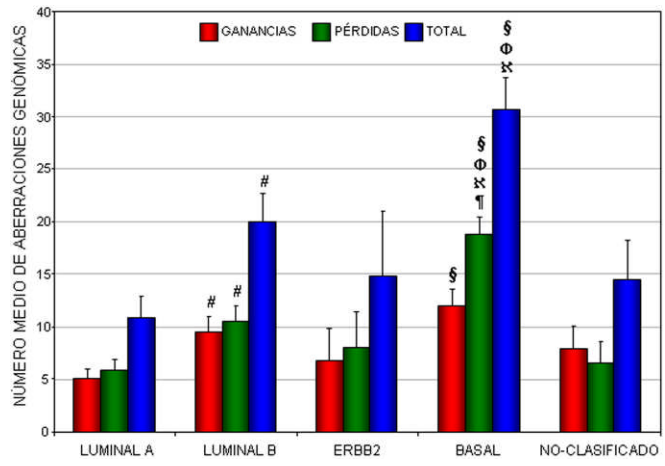


Figura 24. Número medio de aberraciones genómicas por subtipo IHQ. Las diferencias significativas (P≤0'05 en la prueba de U de Mann-Whitney) se muestran en diferentes caracteres dependiendo de la comparación: basal frente a luminal A (§), frente a luminal B (Φ), frente a no-clasificado (X) y frente a ERBB2 (¶), y luminal B frente a luminal A (#)

A continuación, calculamos la frecuencia de cambios genómicos en las diferentes regiones cromosómicas diseñando así el perfil de aberraciones genómicas por cada subgrupo de IHQ (Figura 25). Las aberraciones más recurrentes (con una frecuencia>50%) en el subtipo luminal A fueron las ganancias de 1q y 16p, y las pérdidas de 11q23 y 16q. Los tumores de fenotipo luminal B mostraron alteraciones frecuentes tales como -8ptel-p12, +8q21-qtel, -11q23.3-qtel, -14q31, +16p, -16q, +20q13.13-qtel, ó -22q. Dado que el subtipo ERBB2 se compuso únicamente de cuatro tumores, el perfil de aberraciones genómicas no resultó ser muy informativo, si bien todos los tumores presentaron ganancia de la región 17q12-q21 (locus de *ERBB2*). Por su parte los tumores del subtipo basal presentaron un claro patrón de alta inestabilidad genómicas mostrando altas recurrencias de aberraciones tales como: -3p21-p13, +3q25.1-q26, -4p, -4q22.1-qtel, -5q, -8ptel-p12, +8q22.1-qtel entre otras. Finalmente, los tumores "no clasificados" se caracterizaron por tener un nivel intermedio de inestabilidad genómica comparado con los subtipos luminales A y B. Las únicas dos aberraciones genómicas con una frecuencia superior al 50% en estos tumores fueron +1q32.2 y 8q21.12-qtel. Al comparar las frecuencias de alteraciones genómicas entre los diferentes subtipos IHQ, las únicas diferencias estadísticamente significativas se registraron en la comparación de los tumores basales frente a los tumores "no-basales" (Figura S 2).

Figura 25. Patrones de aberraciones genómicas en cada subtipo IHQ de cáncer de mama familiar. Rojo y verde indican frecuencias de ganancia y pérdidas, respectivamente. Las aberraciones genómicas con una frecuencia mayor a 50% se desglosan al lado de cada subtipo IHQ.

Las amplificaciones de alto nivel fueron más frecuentes en los subtipos luminal B, basal y ERBB2 que en el subtipo luminal A (*P*=0'021, 0'036 y 0'042, respectivamente) (Figura 26A). Algunas de estas aberraciones presentaron una tendencia a ser específicas de cada subtipo, así la amp20q13 fue más frecuente en tumores luminal B, las amplificaciones de 6p22 y 13q34 en el subtipo basal y, como cabía esperar, la amp17q12-q21 en los tumores ERBB2 (Figura 26B).

En resumen, con este estudio se profundizó en la heterogeneidad tanto inmunohistoquímica como genómica que los tumores de mama familiares presentan. Agrupamos el cáncer de mama familiar en cinco subgrupos que resultaron asemejarse a los previamente descritos en cáncer de mama esporádico. En este agrupamiento, si bien los tumores *BRCA1* se asociaban a un fenotipo basal, resulta digno de destacar que no todos los tumores *BRCA1* presentaban este fenotipo. Por otro lado, los tumores *BRCA2* y BRCAX también mostraron una variedad de subtipos demostrando que no se tratan de grupos completamente homogéneos. Una vez agrupadas las muestras familiares en

diferentes subtipos IHQ, fuimos capaces de discernir diferentes patrones de aberraciones genómicas asociados con cada uno de los subtipos IHQ, lo que señala la posible existencia de rutas de evolución tumoral diferentes entre estos subtipos. Estas rutas no solo serían únicas en el cáncer de mama familiar sino que, dadas las similitudes con los subtipos de cáncer de mama esporádico y sus patrones de aberraciones genómicas previamente descritos, deberían considerarse comunes a ambas clases de cáncer de mama y ser objeto de futuros estudios.

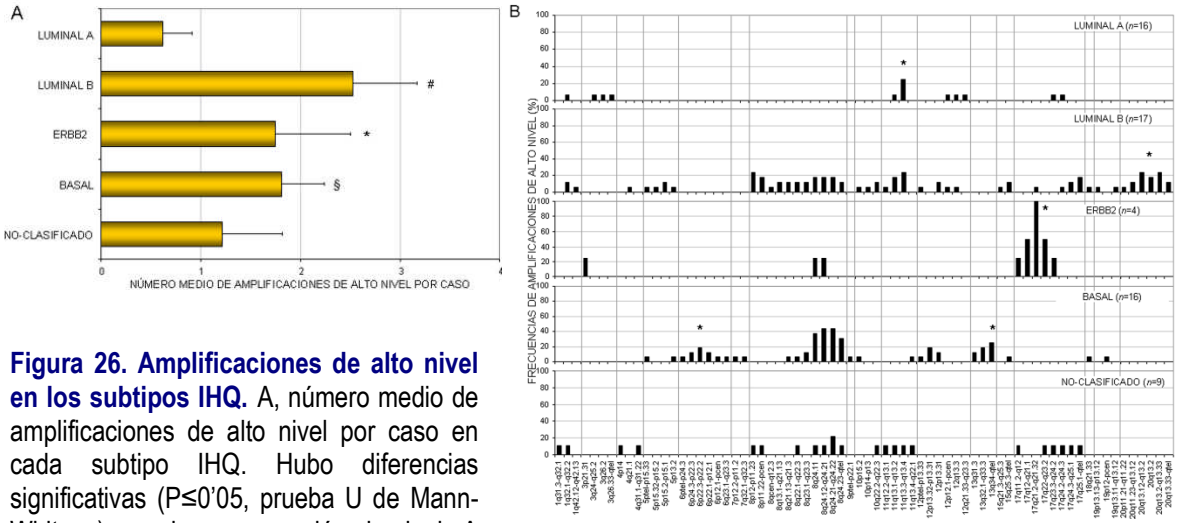


Figura 26. Amplificaciones de alto nivel en los subtipos IHQ. A, número medio de amplificaciones de alto nivel por caso en cada subtipo IHQ. Hubo diferencias significativas (P≤0'05, prueba U de Mann-Whitney) en la comparación luminal A frente a luminal B (#), frente a ERBB2 (*) y frente a basal (§). B, frecuencias de amplificaciones de alto nivel en cada subtipo IHQ. Los sitios cromosómicos de amplificación se muestran en el eje de las X. Las líneas verticales separan cromosomas. Los asteriscos (*) señalan regiones de amplificación específicas de cada subtipo.

RESULTADOS PARTE IV

Caracterización de amplificaciones de alto nivel de dos regiones cromosómicas en cáncer de mama familiar

La amplificación 8p11-p12 se define como un evento molecular complejo compuesto por múltiples regiones mínimas de amplificación y genes candidatos. Hemos estudiado su incidencia, su patrón molecular y su valor clínico en el cáncer de mama familiar. Por cCGH, hemos descrito la amplificación en un 11'25% de casos familiares (3 BRCA1*, 3* BRCA2 *y 3 BRCAX, de 80 tumores). Por aCGH de alta resolución del cromosoma 8, definimos 2 regiones mínimas de amplificación comunes solapantes con las descritas en tumores esporádicos. De este modo, confirmamos la complejidad molecular de esta región cromosómica y la existencia común de esta amplificación en tumores esporádicos y familiares. Por último, asociamos esta aberración genómica con una mayor proliferación (Ki-67) y expresión de ciclina E, lo que apoya la asociación con un peor pronóstico citado con anterioridad en cáncer de mama esporádico.*

Por su parte, la amplificación 13q34 ha sido poco descrita en el cáncer de mama. En nuestros análisis conjuntos de aCGH e IHQ, la amplificación se asoció a tumores RE-negativos y basales. Un examen detallado de su patrón molecular nos muestra una región mínima de amplificación de 4 Mb y un total de 42 genes (de los que 8 son firmes candidatos). Mediante FISH en matrices de tejido esporádicos y familiares, aumentamos el número de muestras aunque la frecuencia fue similar (~5%). Nuestros análisis IHQ señalan TFDP1 *como protagonista por su asociación entre número de copias y expresión proteica. Sin embargo, más estudios son necesarios.*

4. CARACTERIZACIÓN DE AMPLIFICACIONES DE ALTO NIVEL DE DOS REGIONES CROMOSÓMICAS EN CÁNCER DE MAMA FAMILIAR

Uno de los objetivos de la presente tesis fue la definición genómica de regiones cromosómicas que se ven afectadas con amplificaciones de alto nivel en cáncer de mama familiar. Para ello, se abordaron dos estudios diferentes que se centraron en las regiones cromosómicas 8p11-p12 y 13q34. La primera de ellas representa una región objeto de múltiples estudios en cáncer de mama esporádico (Garcia *et al.*, 2005; Gelsi-Boyer *et al.*, 2005; Pole *et al.*, 2006; Prentice *et al.*, 2005; Ray *et al.*, 2004), mientras que la segunda es una región menos conocida (Abba *et al.*, 2007). Ninguna de ellas había sido previamente descrita en cáncer de mama familiar.

4.1. ANÁLISIS GENÓMICO DE LA AMPLIFICACIÓN 8p11-p12 EN CÁNCER DE MAMA FAMILIAR

Como hemos mencionado anteriormente, la amp8p11-p12 se ha descrito en torno a un 10-15% en cáncer de mama esporádico (Adelaide *et al.*, 1998; Courjal and Theillet, 1997; Theillet *et al.*, 1993), si bien su frecuencia se puede ver incrementada hasta un 25% en análisis recientes con aCGH (Garcia *et al.*, 2005; Prentice *et al.*, 2005).

Durante estos últimos años, varios análisis de aCGH han tratado de definir esta región y describir genes relevantes dentro de ella (Garcia *et al.*, 2005; Gelsi-Boyer *et al.*, 2005; Prentice *et al.*, 2005).

En nuestro análisis de las amplificaciones por cCGH, la región 8p11-p12 estaba amplificada en un 11’25% (9 casos de 80 muestras familiares) (Melchor *et al.*, 2005). Las muestras que presentaron amp8p11-p12 fueron 3 tumores *BRCA1*, 3 *BRCA2* y 3 BRCAX, y un ejemplo del patrón de cCGH del cromosoma 8 se detalla en la Figura 27B.

4.1.1. Caracterización genómica de la amplificación 8p11-p12

Para abordar la definición genómica de esta amplificación, utilizamos una plataforma de aCGH específica del cromosoma 8 (ver Material y Métodos 5.2). El patrón

genómico de cada uno de los casos para la región 8p11-p12 se muestra en la Figura 27A.

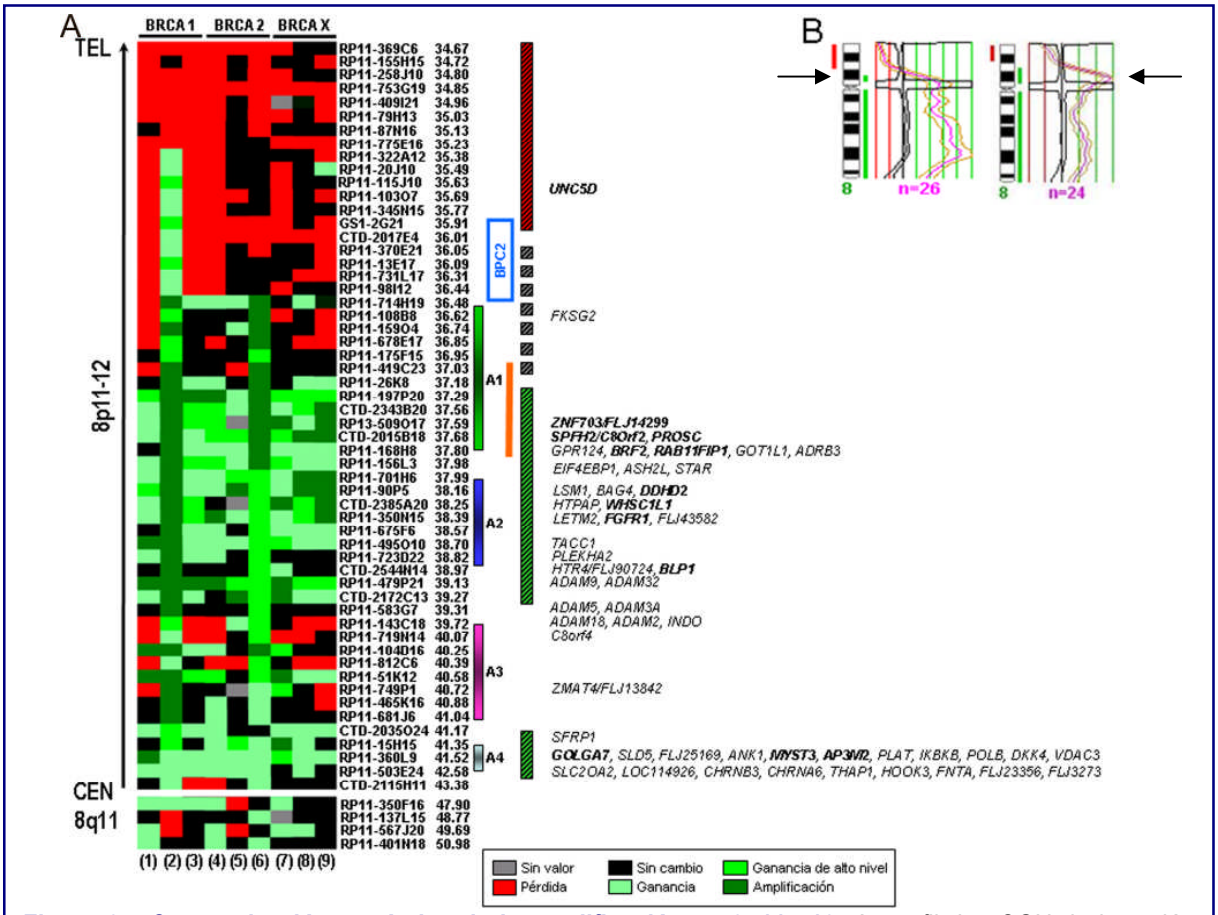


Figura 27. Caracterización genómica de la amplificación en 8p11-p12. A, perfil de aCGH de la región 8p11-p12. Las 9 muestras se representan en tres columnas: 3 BRCA1 (1-3), 3 BRCA2 (4-6) y 3 BRCAX (7-9). Cada fila muestra un clon del array, los cuales se disponen de acuerdo a su posición en la versión 35 del "NCBI Build" desde la más distal (clon de arriba) hasta los más centroméricos (clones de abajo). La posición media (Mb) de cada clon se representa al lado de su nombre, así como los genes que cubre el clon. Los colores muestran los valores discretos de acuerdo al ratio aCGH: rojo indica pérdida (ratio<0'8), negro implica que no hay cambio genómico (0'8-1'2) y una escala de verde para representar distintos niveles de ganancias. El verde claro muestra ganancias simples (1'2-1'5), el verde intermedio significa ganancias de alto nivel (1'5-1'75) y el verde oscuro representa amplificaciones (>1'75). Los cuadros grises corresponden a clones cuyos datos se han rechazado durante las pruebas de calidad para la intensidad de señal y la reproducibilidad de las réplicas. Los genes en negrita son aquellos cuya correlación entre amplificación y sobreexpresión es buena y se ha descrito previamente. Las barras verticales coloreadas junto a los clones señalan los 4 núcleos de amplificación o amplicones (A1, A2, A3, A4) descritos por Gelsi-Boyer y cols. (Gelsi-Boyer et al., 2005). La barra naranja define el amplicón de 1 Mb descrito en otro estudio previo (Garcia et al., 2005). El cuadro azul representa uno de las regiones de rotura descrita previamente (BPC2) y próxima al gen UNC5D (Gelsi-Boyer et al., 2005). Finalmente, las barras coloreadas y rayadas de la derecha representan las regiones de pérdidas distales a los puntos de rotura (barra roja), la zona donde se producen la mayoria de las roturas (barras grises discontinuas) y las 2 regiones de ganancia/amplificación que describimos (barras verdes). B, ejemplo del patrón por cCGH de dos de las muestras de nuestro análisis previo que presentaban la amplificación en 8p11-p12 (señalada con las flechas) (Melchor et al., 2005) y que se incluyeron en el presente estudio.

Las caracterizaciones previas de esta región en líneas y muestras de cáncer de mama esporádico definieron dos regiones habituales de rotura cromosómica, las cuales

delimitaban pérdidas genómicas hacia el telómero y ganancias-amplificaciones genómicas hacia el centrómero. La región de roturas más cercana al telómero (BPC1) se asociaba con reordenamientos del locus *NRG1* (31'38-32'70 Mb) (Gelsi-Boyer *et al.*, 2005; Huang *et al.*, 2004; Pole *et al.*, 2006), mientras que la región de roturas más centromérica (BPC2) se situaba próxima a los genes *NRG1* y *UNC5D* (Garcia *et al.*, 2005; Gelsi-Boyer *et al.*, 2005; Pole *et al.*, 2006). En los casos de cáncer de mama familiar analizados, la mayoría no presentó ningún reordenamiento en la región previamente descrita como BPC1 (datos no mostrados), sino que la mayoría de las roturas cromosómicas ocurrieron entre los BACs CTD-2017E4 (36'01 Mb) y RP11-26K8 (37'18 Mb) (Figura 27A, cuadros grises discontínuos). Esta región de roturas solapa con la previamente descrita como BPC2, lo que indica que esta zona cromosómica es propensa a sufrir roturas tanto en tumores familiares como esporádicos.

En cuanto a las regiones de amplificación, todos los casos mostraron diferentes niveles de ganancias que se agruparon en dos regiones principales (Figura 27A, cuadros verdes rayados de la derecha). Previamente, dos grupos definieron regiones mínimas de amplificación (Garcia *et al.*, 2005; Gelsi-Boyer *et al.*, 2005). Gelsi-Boyer y cols. definieron 4 amplicones (Figura 27A): el más telomérico (A1) se extendía 1'27 Mb y comprendía la región minima de 1 Mb descrita por Garcia y cols. (Garcia *et al.*, 2005), un segundo amplicón (A2) de una longitud de 800 Kb que contiene entre otros genes a *FGFR1*, y finalmente dos amplicones más, A3 y A4, centroméricos a los anteriores y con una longitud de 1'25 Mb y 460 Kb respectivamente. En nuestros casos de cáncer de mama familiar, como mencionamos anteriormente, se registraron dos regiones mínimas de ganancias cuyas longitudes eran de 2'13 Mb y de 1'41 Mb, respectivamente.

La primera región de amplificación comprendió la mitad proximal de A1, todo A2 y una pequeña región de 100 Kb entre A2 y A3 (Figura 27A, cuadro verde rayado superior de la derecha). Esta región genómica contenía los genes: *ZNF703/FLJ14299*, *SPFH2/C8orf2*, *PROSC*, *DDHD2*, *WHSC1L1* y *FGFR1*, todos ellos previamente descritos como genes de interés para análisis funcionales por su buena correlación entre amplificación y sobreexpresión (Garcia *et al.*, 2005; Gelsi-Boyer *et al.*, 2005; Prentice *et al.*, 2005). Esta región también comprendía el amplicón de 1 Mb descrito con anterioridad (Garcia *et al.*, 2005), lo que apoyaba el posible papel de los genes que se localizan en esa región.

La segunda de las regiones de amplificación (Figura 27A, cuadro verde rayado inferior de la derecha) englobó el núcleo A4 de amplificación, tenía una longitud de 1'41 Mb, mostraba un menor nivel de ganancia y contenía otros genes también descritos como relevantes (*GOLGA7*, *MYST3* y *AP3M2*) (Borrow *et al.*, 1996; Gelsi-Boyer *et al.*, 2005).

Por último, la mayoría de nuestros casos no presentaron amplificación en la región A3, excepto dos muestras (casos 2 y 6) que presentaron amplificaciones largas y continuas desde las posiciones de 35 y 36 Mb hasta el centrómero (Figura 27A). Tampoco hubo diferencias en el patrón y distribución de las amplificaciones entre las distintas clases de cáncer de mama familiar (*BRCA1*, *BRCA2* y BRCAX).

4.1.2. Características inmunohistoquímicas de los casos con amplificación en 8p11-p12

La amp8p11-p12 se ha asociado con una mayor proliferación (mayor grado histológico y sobreexpresión de Ki67) (Gelsi-Boyer *et al.*, 2005) y con un efecto adverso en la supervivencia del cáncer de mama (Gelsi-Boyer *et al.*, 2005; Prentice *et al.*, 2005). Para evaluar las posibles asociaciones clínicas de nuestra serie de casos con la amp8p11-p12, comparamos diferentes marcadores IHQ y variables clínicas entre estos 9 tumores y un conjunto de 68 casos que no presentaron este evento genómico (Tabla 9). Observamos que aquellos tumores con amp8p11-p12 presentaron una mayor expresión de Ki-67 y ciclina E de manera estadísticamente significativa (p=0'013 y p=0'033, respectivamente). También se discernió una tendencia a presentar un mayor grado, una mayor expresión de otros marcadores del ciclo celular (como ciclina A y B1) y una edad temprana de aparición del tumor. Sin embargo, estas diferencias no fueron estadísticamente significativas, debido probablemente al bajo número de casos (Tabla 9). Estas variables clínicas y marcadores IHQ se han asociado con una progresión y proliferación tumoral y un peor pronóstico (Chappuis *et al.*, 2005; Foulkes *et al.*, 2004a; Kuhling *et al.*, 2003; Pavelic *et al.*, 1992; Potemski *et al.*, 2006; Simpson *et al.*, 2005). Por lo tanto, nuestos datos indican que esta región de amplificación podría tener un valor clínico en cáncer de mama familiar, tal y como lo presenta en cáncer de mama esporádico. Cabe destacar, que la región A2 descrita como el amplicón asociado con la mayor agresividad de la enfermedad (Gelsi-Boyer *et al.*, 2005) se registraba amplificado en todos nuestros casos.

Tabla 9. Correlación de la amplificación 8p11-p12 con rasgos clínicos y marcadores de IHQ

	Casos sin amplificación n (%)	Casos con amplificación 8p11-p12* n (%)	P**
Edad (años)			
<44	25 (45'5)	5 (55'6)	NS
≥44	30 (54'5)	4 (44'4)	
Grado histológico			
1	17 (28'3)	1 (11'1)	NS
2	17 (28'3)	2 (22'2)	
3	26 (43'3)	6 (66'7)	
Receptor Estrógenos			
<10	24 (35'8)	4 (44'4)	NS
≥10	43 (64'2)	5 (55'6)	
Receptor Progesterona			
<10	32 (47'8)	5 (55'6)	NS
≥10	35 (52'2)	4 (44'4)	
p53			
<25	51 (77'3)	7 (77'8)	NS
≥25	15 (22'7)	2 (22'2)	
Ki-67			
<20	49 (74'2)	3 (33'3)	**0'013**
≥20	17 (25'8)	6 (66'7)	
Ciclina D1			
<25	32 (51'6)	4 (50'0)	NS
≥25	30 (48'4)	4 (50'0)	
Ciclina D3			
<10	34 (56'7)	5 (71'4)	NS
≥10	26 (43'3)	2 (28'6)	
Ciclina E			
<10	46 (74'2)	3 (37'5)	**0'033**
≥10	16 (25'8)	5 (62'5)	
Ciclina A			
<10	23 (37'7)	1 (12'5)	NS
≥10	38 (62'3)	7 (87'5)	
Ciclina B1			
<10	41 (68'3)	3 (42'9)	NS
≥10	19 (31'7)	4 (57'1)	
p16			
<50	22 (40'0)	3 (37'5)	NS
≥50	33 (60'0)	5 (62'5)	
p21			
<10	35 (57'4)	2 (25'0)	NS
≥10	26 (42'6)	6 (75'0)	
p27			
<50	26 (42'6)	4 (50'0)	NS
≥50	35 (57'4)	4 (50'0)	

*Casos cuya amplificación de 8p11-p12 se definió por un estudio previo de cCGH y que cuyo patrón de aCGH se ha analizado en el presente estudio.
**Valores de *p* definidos por la prueba de chi cuadrado de Pearson. NS, no significativo.

En resumen, con el presente estudio hemos descrito y analizado en detalle la amp8p11-p12 por primera vez en cáncer de mama familiar. Describimos dos regiones comunes de amplfiicación que solapan en su mayoría con algunos de los amplicones descritos con anterioridad en cáncer de mama esporádico. También hemos descrito una región de roturas centromérica al gen *UNC5D*, similar a lo descrito en tumores

esporádicos. Por lo tanto, nuestros hallazgos en un grupo selecto de tumores familiares confirman la complejidad molecular de la región cromosómica 8p11-p12 y sugieren que estas alteraciones, y probablemente los genes que se localizan en ellas, son comunes al cáncer de mama, independientemente de la clase tumoral. Además, se asoció la presencia de esta amplificación con una mayor proliferación (Ki67) y expresión de ciclina E, lo cual viene a apoyar el valor clínico de esta aberración descrito previamente en los tumores esporádicos.

4.2. ANÁLISIS Y DEFINICIÓN DE LA AMPLIFICACIÓN 13q34 EN EL CÁNCER DE MAMA FAMILIAR Y ESPORÁDICO

A diferencia de 8p11-p12, la amp13q34 no ha sido objeto de tantos estudios. Se trata de un evento genómico descrito con una baja frecuencia en carcinomas de células escamosas (Shinomiya *et al.*, 1999), carcinoma adrenocortical (Dohna *et al.*, 2000), o hepatocarcinomas (Yasui *et al.*, 2002), entre otros. En lo que respecta al cáncer de mama, solo dos trabajos se han centrado en su caracterización. El primero de ellos fue publicado en 1998 y analizó la frecuencia de amplificación y sobreexpresión de un gen candidato localizado en 13q34: *CUL4A* (Chen *et al.*, 1998b). Por su parte, el segundo de ellos es más reciente y fue publicado durante el desarrollo del presente análisis. Los autores describieron una amplificación y sobreexpresión de un conjunto de genes, localizados en una región homóloga a la región cromosómica humana 13q34, en tumores de mama de modelos murinos. Posteriormente, observaron también este patrón de sobreexpresión en análisis de expresión de cáncer de mama en muestras humanas ya publicados. De este modo, sugirieron *CUL4A*, *LAMP1*, *TFDP1* y *GAS6* como los genes diana de la sobreexpresión y amplificación de esta región en el cáncer de mama (Abba *et al.*, 2007).

En nuestro análisis por cCGH, la región 13q31-q34 estaba amplificada en un 5% (4 de 80 muestras familiares) (Figura 28A). Nuestros posteriores estudios con aCGH confirmaron la presencia y frecuencia de esta amplificación con leves diferencias respecto a la clase de tumor (Tabla 6). Además, esta aberración genómica se asociaba a tumores RE-negativos (ver Resultados 2.4) y basales (ver Resultados 3.2 y Figura 26). Si bien presentaba una frecuencia inferior a otras amplificaciones más estudiadas (8p11p12, 8q24, 17q12-q21…), su especial recurrencia en los tumores basales y la ausencia de una previa caracterización de la región nos llevó a centrar nuestros esfuerzos en la misma.

4.2.1. Caracterización genómica de la amplificación 13q34

A tenor de los perfiles de amplificación ofrecidos por la cCGH, la amplificación de la región 13q31-q34 podía presentar dos perfiles diferentes basados en un inicio de la amplificación más centromérico o telomérico (Figura 28A, comparar casos 03T159 con el resto de los casos). Para profundizar en el patrón molecular de esta amplificación, utilizamos nuestros datos cosechados a partir de la plataforma de 1Mb de aCGH. De este modo, confirmamos la amplificación en los cuatro casos anteriores (Figura 28B, casos 1-4) y la encontramos también en un caso esporádico (caso 6) y en la línea celular MDA-MB-157 (caso 7). Como en el perfil de cCGH, el cromosoma 13 aparece alterado pudiendo presentar dos patrones diferentes: a) una pérdida desde el centrómero hasta los alrededores de la región 13q31 en la que se inicia la ganancia-amplificación (Figura 28B, casos 1, 3, 4, 6 y 7), ó b) una ganancia-amplificación que se inicia en los alrededores de la región 13q21 sin ninguna pérdida genómica en la región vecina (Figura 28B, casos 2 y 5). Estos patrones sugieren la existencia de dos mecanismos moleculares diferentes como la causa biológica de esta amplificación genómica. Un análisis detallado de la región 13q31-q34 nos mostró que la mínima región común de amplificación se localizaba en 13q34, independientemente del patrón cromosómico general (Figura 28C). Esta región mínima se extiende un total de 1’6 Mb (desde RP11-375A8 hasta el telómero) y presenta un total de 29 genes (incluyendo genes hipotéticos de función desconocida), de los que 7 resultan de especial interés por su relación con procesos tumorales: *ATP11A*, *CUL4A*, *LAMP1*, *TFDP1*, *GAS6*, *RASA3* y *CDC16*. Muchos de estos genes coinciden con los propuestos como candidatos en anteriores estudios (Abba *et al.*, 2007; Chen *et al.*, 1998b).

Antes de proceder al análisis de expresión de los genes candidatos, decidimos realizar unas pruebas FISH en unas matrices de tejido de tumores esporádicos y familiares de una serie parcialmente independiente (compuesta de 24 BRCA1, 21 BRCA2, 96 BRCAX y 259 muestras esporádicas), así como en las líneas celulares que presentaban indicios de amplificación por aCGH y/o cCGH (HCC-1937, MDA-MB-157 y MDA-MB-157). Con este procedimiento, pretendimos: 1) validar la existencia de amplificación con unos valores concretos del número de copias y 2) aumentar el número de casos para poder definir mejor el patrón molecular. A tal efecto, preparamos una sonda FISH con clones cercanos al centrómero y clones situados en la región 13q34 (Material y Métodos 3.4.3).

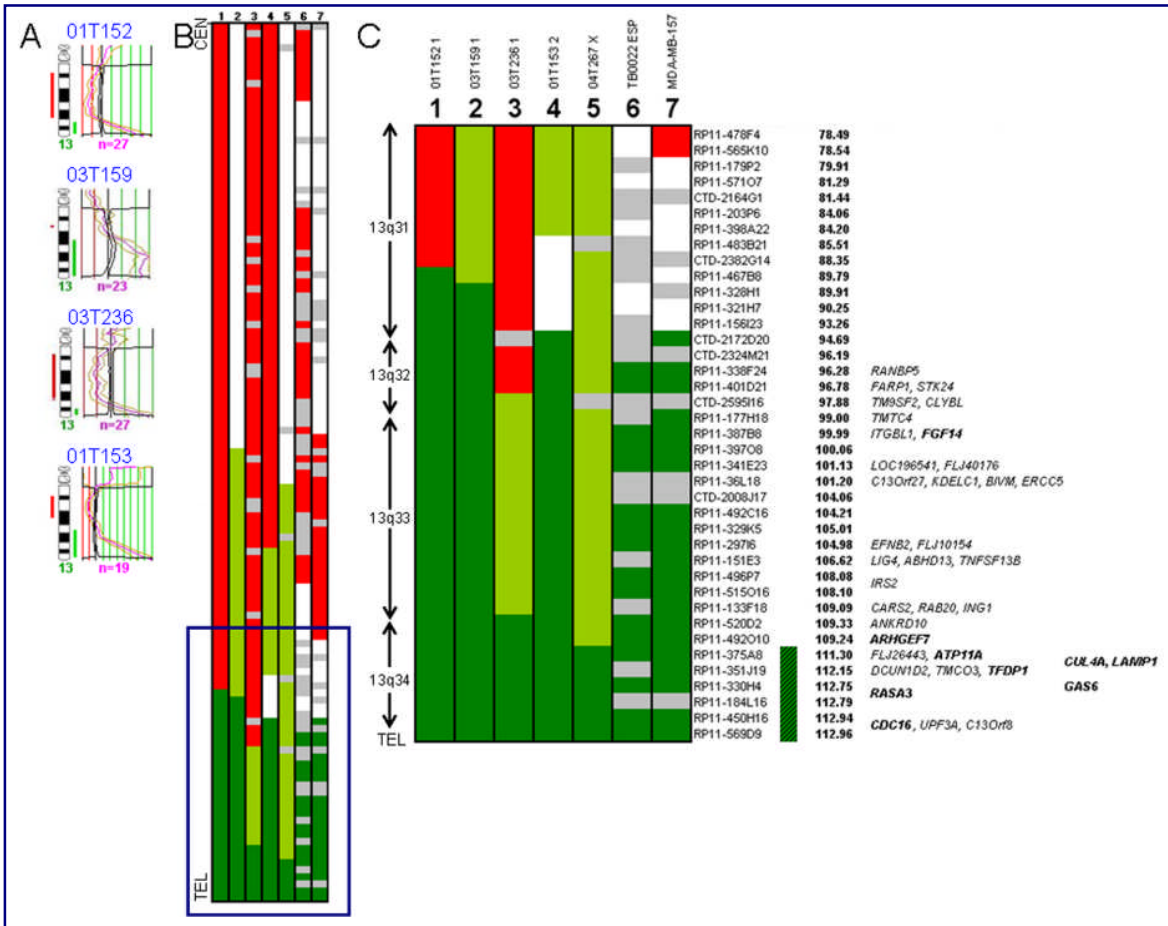


Figura 28. Caracterización genómica de la amplificación 13q34. A, perfiles de cCGH de los cuatro casos que presentaban la amplificación 13q34 en nuestro primer estudio (Melchor et al., 2005). B, patrón genómico del cromosoma 13 en 3 BRCA1 (1-3), 1 BRCA2 (4), 1 BRCAX (5), 1 esporádico (6) y una línea celular (7); hacia arriba el centrómero y hacia abajo el telómero del cromosoma 13. C, patrón genómico de la región 13q31-q34 (recuadrada en la parte B de la figura). Cada fila representa un clon del array, dispuestos en función a su posición enla versión 35 del "NCBI Build". Se muestra su posición media (Mb), así como los genes que cubren cada clon desde la región 13q32 (primera región mínima de ganancia). Los colores muestran el nivel del número de copias: rojo (pérdida), blanco (no cambio), verde claro (ganancia), verde oscuro (amplificación) y gris (sin disponibilidad de datos). Los genes en negrita son aquellos resaltados en análisis previos de esta amplificación. La barra verde rayada representa la región mínima de amplificación entre los casos hibridados (13q34).

Establecido el umbral de amplificación como la presencia de la sonda 13q34 de tres veces la región considerada control (13q12.11), se validó la amplificación en los casos estudiados por el aCGH en secciones independientes de tejidos (datos no mostrados). Asimismo, se realizó la prueba FISH en líneas celulares cuyos patrones de aCGH o cCGH hacían suponer una amplificación: la línea MDA-MB-157 presentó una amplificación de la región (>6 copias) que además se repartía en diferentes cromosomas derivados (Figura 29A); la línea MDA-MB-436, que presentaba amplificación-ganancia de la región en un reciente estudio (Neve *et al.*, 2006), resultó tener ganancia de dos copias (Figura 29B);

finalmente, la línea celular con mutación en *BRCA1* HCC1937 y cuyo perfil de cCGH hacía inducir unos valores de amplificación presentó una notable heterogeneidad celular con polisomías del cromosoma 13 y ganancias, que no amplificación, de la región 13q34 (Figura 29C).

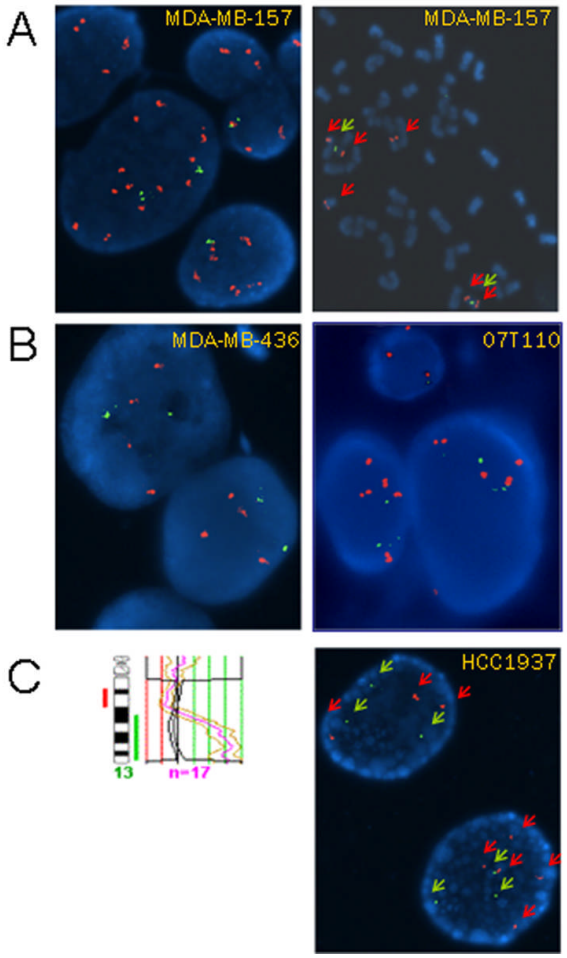


Figura 29. Ensayos FISH de la región de amplificación 13q34. La sonda se compone de dos cócteles de BACs que cubren la región 13q12.11 en verde (V) y 13q34 en rojo (R), respectivamente. A, en la línea celular MDA-MB-157 donde se aprecian dos señales V y >6 R por núcleo (izquierda) o metafase (derecha). B, en la línea celular MDA-MB-436 donde se aprecian 2V y 4R, y en un caso en parafina 07T110 obtenido en el análisis FISH de matrices de tejido. C, en la línea celular HCC1937 la cual por cCGH parece mostrar amplificación de la región (izquierda) que, sin embargo, se descartaría a partir de la FISH ya que presenta 3V y 3-5R por núcleo (derecho).

Por su parte, el segundo de los objetivos al realizar las pruebas FISH sobre las matrices de tejidos se cumplió. En primer lugar, obtuvimos un resultado FISH analizable en un 87’5% de *BRCA1* (21/24), un 66’7% de *BRCA2* (14/21), 68’7% de BRCAX (66/96) y de un 74’1% de esporádicos (192/259). Aquellos casos en los que la FISH resultó fallida fue debido a la ausencia de tejido, bien porque ya se había agotado o por su pérdida durante el proceso experimental, o por un fallo de la hibridación al ser el tejido demasiado antiguo o estar compuesto por excesivo tejido conectivo que impide la correcta hibridación. En segundo lugar, la FISH nos permitió fijar la frecuencia de los tumores de mama que amplificaban 13q34 en un 4’43% (13/293) si bien la incidencia se presentaba ligeramente superior en los tumores *BRCA1* (9’52%, 2/21) que en el resto de clases tumorales: 0/14 *BRCA2*, 4’76% de los BRCAX (3/66) y 4’17% de los esporádicos (8/192).

De este modo, conseguimos un mayor número de casos con los que perfeccionar la definición del patrón genómico de esta amplificación (p.ej. el caso 5 de la Figura 28 es un tumor BRCAX registrado como amplificado en el estudio FISH). Durante la redacción de la presente tesis, se están llevando a cabo la hibridación de los casos disponibles adicionales registrados por los análisis FISH (el caso 07T110 de la Figura 29B es un ejemplo). Un total de 5 casos esporádicos serán analizados.

4.2.2. Estudio inmunohistoquímico de un gen candidato de la región 13q34

Como se ha mencionado con anterioridad, un total de 7 genes localizados en 13q34 podrían ser catalogados como los genes diana de este evento genómico. A tenor de los estudios previos de esta región, el gen *TFDP1* puede erigirse como el principal candidato dada su alta correlación de niveles de amplificación-sobreexpresión y su papel como factor de transcripción de promotores del ciclo celular (como ciclina E) (Abba *et al.*, 2007; Yasui *et al.*, 2002). Por lo tanto, iniciamos el análisis de expresión de cada uno de los genes candidatos estudiando *TFDP1* mediante pruebas de inmunohistoquímica (ver Tabla 3).

Tabla 10. Datos de expresión de TFDP1 en el cáncer de mama familiar y esporádico.

Expresión de TFDP1	Total	BRCA1	BRCA2	BRCAX	Esporádico
Negativa	147 (48'7)	13 (61'9)	8 (42'1)	59 (68'6)	67 (38'1)
Positiva (≥25%)	155 (51'3)	8 (38'1)	11 (57'9)	27 (31'4)	109 (61'9)
Levemente sobreexpresado (25-60%)	86 (28'5)	6 (28'6)	9 (47'4)	18 (20'9)	53 (30'1)
Altamente sobreexpresado (≥60%)	69 (22'8)	2 (9'5)	2 (10'5)	9 (10'5)	56 (31'8)

Las casillas presentan el número de casos y el porcentaje en paréntesis sobre el total de muestras por cada clase dispuesta en cada columna. Los niveles de positividad y de sobreexpresión se definieron a partir de las medianas de todos los valores y de los valores positivos, respectivamente.

La expresión de TFDP1 fue analizada de una manera particular en los casos que presentaban la amp13q34 en el análisis por aCGH y de una manera general en las matrices de tejido. A partir del análisis de su expresión en la serie de muestras parcialmente independientes que componían las matrices de tejido, establecimos el nivel de sobreexpresión en función del valor de la mediana de todos los casos, lo que a la postre resultó en al menos un 25% de células positivas. Asi mismo, entre los casos con sobreexpresión definimos los levemente sobreexpresados (un porcentaje de positividad entre 25-60%) y los altamente sobreexpresados (>60%). El número y porcentaje de casos

negativos y positivos para la expresión de TFDP1 por cada una de las clases tumorales se muestra a continuación (Tabla 10).

Tabla 11. Correlación entre el número de copias de la región 13q34 y el nivel de expresión de TFDP1.

Número de copias 13q34	Expresión de TFDP1				
	TFDP1 (2 niveles)		TFDP1 (3 niveles)		
13q34	Negativo	Positivo	Negativo	Levemente sobreexpresado	Altamente sobreexpresado
No Amplifica	115 (48'3)	123 (51'7)	115 (48'3)	74 (31'1)	49 (20'6)
Amplifica	1 (7'7)	12 (92'3)	1 (7'7)	2 (15'4)	10 (76'9)
	P=0'004*			*P*=0'000*	
	TFDP1 (2 niveles)		TFDP1 (3 niveles)		
13q34	Negativo	Positivo	Negativo	Levemente sobreexpresado	Altamente sobreexpresado
Perdida o No cambio	110 (49'8)	111 (50'2)	110 (49'8)	69 (31'2)	42 (19'0)
Ganancia	5 (29'4)	12 (70'6)	5 (29'4)	5 (29'4)	7 (41'2)
Amplificación	1 (7'7)	12 (92'3)	1 (7'7)	2 (15'4)	10 (76'9)
	P=0'004*			*P*=0'000*	

Cada casilla muestra el número de casos y el porcentaje en paréntesis en función de la dotación genómica de 13q34.
*Valores de *P* calculados a partir de la prueba chi cuadrado de Pearson.

Cuando contrastamos si había una correlación entre los niveles del número de copias medidos por FISH y el de expresión proteica obtenidos a partir del ensayo de IHQ, observamos que había una correlación positiva. Esta asociación fue estadísticamente significativa independientemente de: a) si se establecían dos niveles de expresión (negativa/positiva, p=0'004) o tres niveles (negativa/levemente/altamente sobreexpresado, p=0'000) y b) del nivel de número de copias: no amplificación/amplificación o pérdida-no-cambio/ganancia/amplificación (Tabla 11). Cabe destacar que el 92'3% de los tumores que amplifican 13q34 sobreexpresan la proteína TFDP1 y, más allá, el 76'9% de los que amplifican expresan un alto nivel de proteína. Por el contrario, de aquellos tumores que no amplifican, sólo el 51'7% sobreexpresan la proteína y, concretamente, el 20'6% total lo hacen en un alto nivel (Tabla 11). De este modo, podemos afirmar que un mayor número de copias de la región 13q34 está asociado a una mayor expresión proteica de *TFDP1*, un gen localizado dentro de la región. Sin embargo, la amp13q34 no resulta ser el único

mecanismo por el cual un tumor sobreexpresa la proteína estudiada, dado que existen casos que sin presentar amplificación tienen una sobreexpresión.

Se muestran ejemplos de la tinción IHQ de la proteína TFDP1 en la Figura 30.

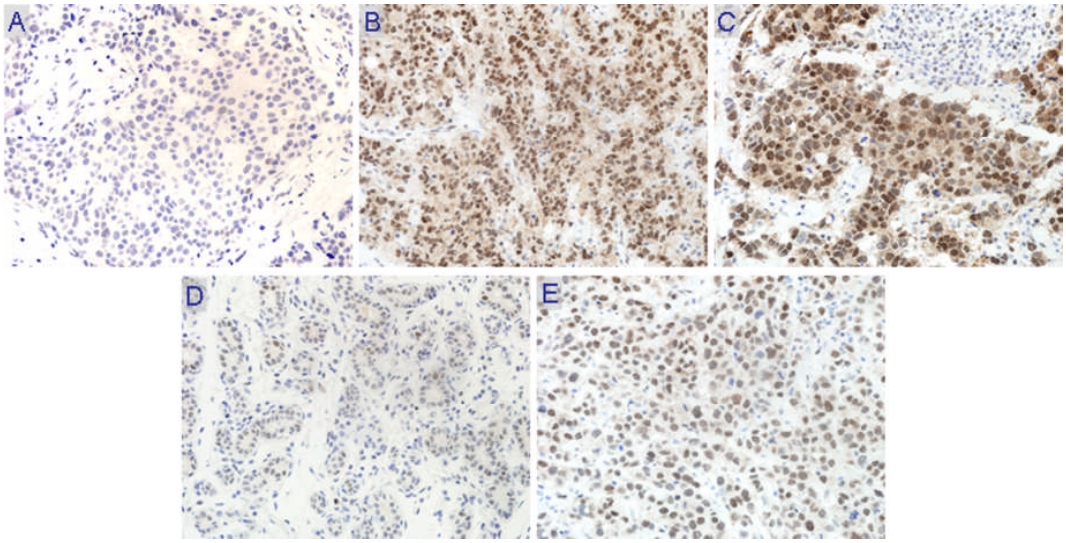


Figura 30. Tinción inmunohistoquímica de TFDP1. A, caso con expresión negativa (<25% de células positivas). B-C, casos con expresión positiva (>25% de células positivas), adviértase la población celular de linfocitos en el margen superior derecha de la figura C, la cual es negativa para TFDP1. El caso C presentó amplificación de 13q34 por FISH y aCGH (caso 5 de la Figura 28B). D-E, porciones de tejido normal (D) y tumoral (E) de un caso con amplificación 13q34 por aCGH (caso 2 de la Figura 28B) y validada por FISH. La región normal (D) muestra negatividad para TFDP1, mientras que la región tumoral (E) muestra positividad para TFDP1.

Durante la redacción de la presente tesis, se están abordando estudios de reacción de la cadena de polimerasa a partir de ARN retrotranscrito (RT-PCR) procedente de los tumores que presentaban la amplificación, de tumores sin alteración genómica en 13q34 y de tejidos normales. Todos ellos son tejidos embebidos en parafina por lo que el proceso es más complejo que si fueran tejidos congelados. De este modo, deseamos estudiar los niveles de expresión de ARNm de cada uno de los siete genes candidatos y, posteriormente, estudiar los niveles de expresión proteica mediante ensayos IHQ como el realizado hasta el momento con *TFDP1* de aquellos cuya expresión de ARNm resulte importante. Sin embargo, todavía no se han cosechado ningún resultado para poder reflejarlo en la presente tesis a excepción de lo presentado aquí con la proteina TFDP1.

4.2.3. Asociación de la amplificación 13q34 y de la sobreexpresión de TFDP1 con otros marcadores IHQ y el grado histológico

Definida la asociación existente entre el número de copias de la región 13q34 y el nivel de expresión de uno de sus genes candidatos (*TFDP1*), nos interesamos por el resto de variables clínicas y características IHQ que pudieran caracterizar a aquellos tumores con amp13q34 (por aCGH y/o FISH) o sobreexpresión de TFDP1. Por lo tanto, realizamos los contrastes estadísticos con aquellos casos de los que se disponía de información clínica e IHQ (de los marcadores desglosados en la Tabla 3) obtenida a lo largo de nuestros anteriores estudios (Honrado *et al.*, 2007; Honrado *et al.*, 2005b; Palacios *et al.*, 2005b; Palacios *et al.*, 2003). En la Tabla 12 se muestran el grado histológico y los 19 marcadores IHQ que tuvieron unos valores de *P* interesantes.

Observamos que los tumores que amplifican 13q34 (valorados por aCGH y/o por FISH) se caracterizaron por presentar un mayor grado; una negatividad para RE, RP, CCND1, RB, p16, CK8 y CAM5.2; y positividad para Ki-67, EGFR, P-caderina, g-catenina, CCNE, CCNB1, SKP2, survivina, vimentina y CK5 (Tabla 12). La asociación con estos marcadores podría explicarse porque la amp13q34 se describió asociada a los tumores con fenotipo basal (Figura 26B). De hecho, la mayoría de estos marcadores son los que caracterizaron al fenotipo basal en uno de nuestros anteriores estudios (Figura 23 y Tabla 8).

Por el contrario, los tumores que sobreexpresan TFDP1 se asociaron a una mayor positividad para CCNE, CCNB1, p16 y RAD50; y una tendencia a una mayor expresión de E2F6, MDM2 y p27 (Tabla 12). Estas diferencias con respecto a los marcadores IHQ asociados a los tumores que amplifican 13q34 indican que el fenómeno de la sobreexpresión de TFDP1 no es exclusivo de los tumores con fenotipo basal, a diferencia de la amp13q34. Es decir, que la sobreexpresión de TFDP1 es universal en cáncer de mama y puede conseguirse por mecanismos diferentes al de la amplificación, mecanismo éste que adquiere especial importancia en los tumores basales. Cabe destacar que tanto los tumores que amplifican 13q34 como los que sobreexpresan TFDP1 se caracterizan por una mayor expresión de CCNE y CCNB1. Esto puede deberse a que el factor de transcripción DP1 se asocia con E2F1 para promover la expresión de proteínas relacionadas con el ciclo celular y concretamente con el paso a la fase S del mismo como, por ejemplo, ciclina E. De

hecho, la asociación entre la expresión de TFDP1 y de CCNE ya ha sido descrita (Yasui *et al.*, 2002).

Tabla 12. Asociación de la amplificación 13q34 y de la sobreexpresión de TFDP1 con otros marcadores IHQ y el grado histológico.

Variable	Amplificación de 13q34			Expresión de TFDP1		
	Negativo	Positivo	P	Negativo	Positivo	P
Grado						
1	42 (32'1)	0		12 (32'4)	27 (33'3)	
2	40 (30'5)	0	**0'002**	11 (29'7)	22 (27'2)	NS*
3	49 (37'4)	8 (100)		14 (37'8)	32 (39'5)	
RE						
Negativo	56 (35'4)	8 (88'9)	**0'002**	24 (46'2)	35 (38'0)	NS
Positivo	102 (64'6)	1 (11'1)		28 (53'8)	57 (62'0)	
RP						
Negativo	68 (47'2)	8 (88'9)	**0'018**	20 (48'8)	46 (52'3)	NS
Positivo	76 (52'8)	1 (11'1)		21 (51'2)	42 (47'7)	
BCL2						
Negativo	82 (56'9)	8 (88'9)	0'082	29 (70'7)	51 (58'0)	NS
Positivo	62 (43'1)	1 (11'1)		12 (29'3)	37 (42'0)	
Ki-67						
0-5%	68 (47'2)	1 (11'1)		22 (53'7)	35 (39'8)	
6-25%	56 (38'9)	1 (11'1)	**0'000***	13 (31'7)	32 (36'4)	NS*
>25%	20 (13'9)	7 (77'8)		6 (14'6)	21 (23'9)	
EGFR						
Negativo	181 (90'0)	4 (40'0)	**0'000**	94 (91'3)	89 (86'4)	NS
Positivo	20 (10'0)	6 (60'0)		9 (8'7)	14 (13'6)	
P-Caderina						
Negativo	128 (92'1)	3 (33'3)	**0'000**	37 (94'9)	74 (87'1)	NS
Positivo	11 (7'9)	6 (66'6)		2 (5'1)	11 (12'9)	
g-Catenina						
Negativo	106 (79'1)	4 (44'4)	**0'031**	36 (90'0)	69 (79'3)	NS
Positivo	28 (20'9)	5 (55'5)		4 (10'0)	18 (20'7)	
MDM2						
Negativo	108 (78'3)	9 (100)	NS	35 (89'7)	63 (75'0)	0'090
Positivo	30 (21'7)	0		4 (10'3)	21 (25'0)	
Ciclina D1						
Negativo	67 (47'2)	8 (88'9)	**0'018**	17 (42'5)	41 (47'1)	NS
Positivo	75 (52'8)	1 (11'1)		23 (57'5)	46 (52'9)	
Ciclina E						
Negativo	103 (72'5)	2 (22'2)	**0'004**	32 (80'0)	53 (60'9)	**0'042**
Positivo	39 (27'5)	7 (77'8)		8 (20'0)	24 (39'1)	
Ciclina A						
Negativo	61 (43'6)	1 811'1)	0'081	18 (45'0)	40 (46'0)	NS
Positivo	79 (56'4)	8 (88'9)		22 (55'0)	47 (55'0)	
Ciclina B1						
Negativo	111 (80'4)	4 (44'4)	**0'024**	37 (92'5)	61 (73'5)	**0'016**
Positivo	27 (19'6)	5 (55'6)		3 (7'5)	22 (26'5)	
RB						
Negativo	33 (23'9)	6 (66'7)	**0'011**	11 (27'5)	14 (16'5)	NS
Positivo	105 (76'1)	3 (33'3)		29 (72'5)	71 (83'5)	
E2F6						
Negativo	89 (64'5)	4 (44'4)	NS	30 (75'0)	47 (56'6)	0'073
Positivo	49 (35'5)	5 (55'6)		10 (25'0)	36 (43'4)	
P16						
Negativo	53 (39'0)	7 (77'8)	**0'033**	23 (59'0)	31 (36'5)	**0'031**
Positvo	83 (61'0)	2 (22'2)		16 (41'0)	54 (63'5)	
P27						
Negativo	60 (43'2)	6 (66'7)	NS	26 (65'0)	41 (48'2)	0'087
Positivo	79 (56'8)	3 (33'3)		14 (35'0)	44 (51'8)	
SKP2						
Negativo	66 (46'8)	1 (11'1)	**0'043**	24 (60'0)	40 (46'5)	NS
Positivo	75 (53'2)	8 (88'9)		16 (40'0)	46 (53'5)	
Survivina						
Negativo	97 (70'8)	1 (11'1)	**0'001**	30 (75'0)	57 (65'5)	NS
Positivo	40 (29'2)	8 (88'9)		10 (25'0)	30 (34'5)	
CK5						
Negativo	133 (86'4)	3 (33'3)	**0'001**	42 (82'4)	77 (83'7)	NS
Positivo	21 (13'6)	6 (66'6)		9 (17'6)	15 (16'3)	

CK8						
Negativo	28 (19'9)	7 (77'8)	**0'001**	7 (17'5)	23 (26'1)	NS
Positivo	113 (80'1)	2 (22'2)		33 (82'5)	65 (73'9)	
CAM 5.2						
Negativo	43 (44'3)	5 (100)	**0'021**	9 (39'1)	26 (44'8)	NS
Positivo	54 (55'7)	0		14 (60'9)	32 (55'2)	
Vimentina						
Negativo	109 (79'0)	3 (33'3)	**0'006**	32 (80'0)	63 (73'3)	NS
Positivo	29 (21'0)	6 (66'6)		8 (20'0)	23 (26'7)	
RAD50						
Negativo	49 (38'6)	4 (44'4)	NS	19 (50'0)	25 (29'1)	**0'041**
Positivo	78 (61'4)	5 (55'6)		19 (50'0)	61 (70'9)	
XRCC3 citoplasma						
Negativo	57 (42'9)	0	**0'011**	16 (40'0)	39 (44'8)	NS
Positivo	76(57'1)	9 (100)		24 (60'0)	48 (55'2)	

Se representan los valores de P≤0'10 y en negrita aquellos significativos (P≤0'05). NS, no significativo. Los valores de P fueron calculados por medio de la prueba exacta de Fisher a excepción del grado y Ki-67 (*) que se hicieron con pruebas de chi cuadrado de Pearson.

En resumen, con este estudio estamos definiendo una región mínima de amplificación en 13q34 de 1'6 Mb y que contiene un total de 7 genes candidatos. Los estudios IHQ con uno de estos genes, *TFDP1*, nos ha permitido establecer una correlación entre el número de copias de 13q34 y el nivel de sobreexpresión de TFDP1. Si bien la amplificación parece ser característica de los tumores basales, la sobreexpresión de TFDP1 se registra también en el resto de subtipos de cáncer de mama por lo que la amplificación de su región genómica no es el único mecanismo de sobreexpresión. Asimismo, una mayor expresión de TFDP1 conlleva una sobreexpresión de otros genes como CCNE, importantes durante el ciclo celular. Sin embargo, este estudio todavía está en desarrollo y requiere de una mayor definición de la región mínima de amplificación, del análisis de expresión del resto de genes candidatos y del establecimiento de asociaciones con otros marcadores IHQ o variables clínicas siempre que sea posible.

DISCUSIÓN

En esta tesis hemos descrito los resultados obtenidos a partir del análisis del número de copias de ADN en tumores de mama familiar y esporádico. Para ello, hemos empleado técnicas de citogenética molecular basadas en la hibridación genómica comparativa sobre cromosomas (cCGH) y sobre arrays (aCGH). Un total de 27 muestras asociadas a mutación de *BRCA1*, 28 casos *BRCA2*, 38 muestras BRCAX y 19 tumores esporádicos se han repartido entre los distintos estudios abordados.

Al comparar un mismo tumor estudiado por cCGH y aCGH, los patrones de aberraciones genómicas se reprodujeron en su mayoría, aunque la descripción de las alteraciones fue más detallada por aCGH (Figura 31). El aCGH (compuesto de 4.134 clones o BACs que cubren todo el genoma) se confirma de este modo como una técnica con mayor poder resolutivo que la cCGH, siendo las resoluciones límites de ~1 Mb para la primera y de ~10 Mb para la segunda. Un ejemplo de esta mayor resolución es la amplificación de 11q13.3 (locus de *CCND1*) poco frecuente en nuestro estudio de cCGH pero que, sin embargo, se describe más recurrentemente por aCGH. Esto puede ser porque pase desapercibida por cCGH al ser una amplificación de pequeño tamaño y que suele flanquearse de pérdidas del resto de 11q. Otro ejemplo es la amplificación 17q12-q21 de la Figura 31A que, gracias al aCGH, se describe compuesta por dos núcleos de amplificación separados por clones sin alteración (Figura 31B).

Sin embargo, también creemos que la técnica o el análisis de aCGH tienen que ser optimizados, especialmente para evitar problemas relacionados con artefactos que llevan a considerar como ganancias regiones que realmente no están alteradas. En el CNIO, hemos identificado este fenómeno en aCGH independientemente de la plataforma utilizada (array de BACs o de oligos), de la muestra (tumoral o normal, tejido congelado o parafinado) y del laboratorio en el que se desarrolló la hibridación (Blesa *et al.*, en preparación). Un problema similar se describió en cCGH hace una década y se tuvo en cuenta para su correcto análisis (Kirchhoff *et al.*, 1998). En la presente tesis, elaboramos un método de estandarización análogo al empleado por Kirchhoff y cols. para eliminar el efecto de estos artefactos (ver Material y Métodos 5.1.3, Resultados 2.1 ó Figura 11) y, tras ensayos FISH de validación, confirmamos un importante descenso en el número de aberraciones consideradas como falso positivas. Recientemente, un estudio hace mención también a este fenómeno al que llama “olas artefactuales del genoma” e incluso proponen un método estadístico para su tratamiento (Marioni *et al.*, 2007). De este modo, tanto este trabajo

como los desarrollados por nuestro grupo llaman la atención sobre un fenómeno que había pasado inadvertido hasta el momento en la técnica de aCGH, pero que ya había sido considerado en la técnica previa al aCGH: la cCGH. Resolver este problema es de máxima importancia para conseguir una caracterización genómica concisa e inequívoca.

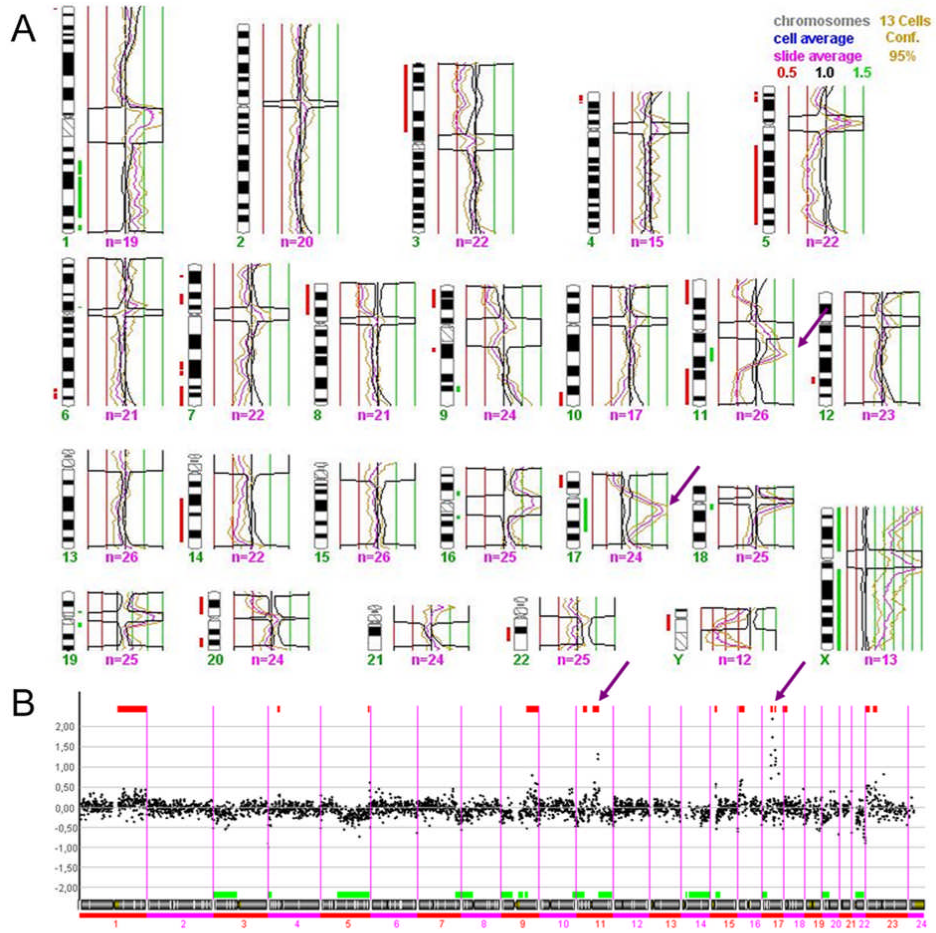


Figura 31. Comparación del patrón de aberraciones genómicas por cCGH y por aCGH en un mismo tumor. A, patrón por cCGH en el que se muestran en barras verticales al lado del idiograma cromosómico las pérdidas (rojo) y las ganancias (verde). B, patrón por aCGH en el que se muestra en barras horizontales las pérdidas (parte inferior y en verde) y ganancias (parte superior y en rojo). Cabe destacar la confirmación de aberraciones entre uno y otro patrón tales como +1q, -3q, -5q, -8p, etc. las cuales quedan todavía más definidas por el aCGH. De hecho, la ganancia de 11q13 y la amplificación de 17q12-q21 en cCGH (A, flechas violetas) quedan refinadas en el patrón de aCGH (B, flechas violetas). Especialmente se observa que 17q12-q21 presenta al menos dos núcleos de amplificación separadas por unos clones no ganados, mientras que en el patrón de cCGH parece una amplificación continua.

Describiremos a continuación nuestros principales hallazgos y los compararemos con la literatura previa. Asimismo, esbozaremos un modelo de inicio y progresión del cáncer de mama integrando varios conceptos como las hipótesis de células troncales cancerígenas y de selección clonal, los subtipos de cáncer de mama en los tumores familiares y esporádicos y la posible influencia de mutaciones en los genes *BRCA*.

1. CARACTERÍSTICAS GENÓMICAS DEL CÁNCER DE MAMA FAMILIAR *BRCA1*, *BRCA2* Y BRCAX

Uno de nuestros objetivos establecer las diferencias genómicas entre cada una de las distintas clases de cáncer de mama familiar (*BRCA1*, *BRCA2* y BRCAX) y el cáncer de mama esporádico. Cabía esperar que tumores asociados a una mutación *BRCA1* ó *BRCA2* desarrollaran un patrón de aberraciones genómicas característico que nos ofreciera la oportunidad de construir y/o validar clasificadores genómicos. Estas herramientas podrían servir en la práctica clínica al definir un tumor como posible *BRCA1* ó *BRCA2*, sugiriendo un estudio mutacional dirigido con el ahorro en tiempo y recursos que ello conlleva. Asimismo, este estudio era pionero en la descripción del patrón genómico de los tumores BRCAX.

1.1. EL PATRÓN DE INESTABILIDAD GENÓMICA DEL CÁNCER DE MAMA ESPORÁDICO Y FAMILIAR *BRCA1*, *BRCA2* Y BRCAX

Nuestro grupo ha logrado profundizar en los últimos años en la caracterización genómica del cáncer de mama familiar a través de estudios de cCGH y aCGH, (Alvarez *et al.*, 2005; Melchor *et al.*, 2005; Melchor *et al.*, 2007c). En todos ellos, describimos una mayor inestabilidad genómica en los tumores *BRCA1/2* que en tumores BRCAX o esporádicos (ver Resultados epígrafes 1 y 2.2.1), confirmando previos estudios de cCGH (Tirkkonen *et al.*, 1997; van Beers *et al.*, 2005; Wessels *et al.*, 2002) y aCGH (Jonsson et al., 2005). El mayor número de aberraciones genómicas se presentó en los tumores *BRCA1*, los tumores *BRCA2* fueron el segundo grupo más inestable y los tumores esporádicos, por su parte, presentaron mayor inestabilidad que los tumores BRCAX pero menor que los tumores *BRCA2* (Figura 18). Esto podría deberse al papel que los genes *BRCA* presentan en la reparación del daño al ADN, ya que una deficiente reparación podría traducirse en un aumento de la inestabilidad genómica.

Hemos encontrado una serie de aberraciones genómicas comunes (>40%) por aCGH en todas las clases tumorales: +1q, +16p13.3, y -8ptel-p12 y -16q (Figura 19). Al compararlo con las aberraciones comunes registradas en nuestro estudio previo de cCGH, las alteraciones del cromosoma 16 eran sustituidas por las aberraciones +8q21-q23 y -11q22-q25. Sin embargo, esta diferencia podía atribuirse a las series de muestras

parcialmente diferentes entre ambos estudios y a la inclusión de una serie de tumores esporádicos en nuestro estudio de aCGH. No obstante, todas estas aberraciones comunes coincidían con las citadas en análisis anteriores de cCGH (van Beers *et al.*, 2005; Wessels *et al.*, 2002), destacando las similitudes con el estudio de van Beers y cols. (van Beers et al., 2005), en el que analizaron un total de 80 regiones cromosómicas definidas de una manera muy similar a la descrita por nosotros con 63 regiones cromosómicas por cCGH (Alvarez *et al.*, 2005; Melchor *et al.*, 2005). Estas aberraciones genómicas comunes a cáncer de mama familiar y esporádico podrían contener genes cruciales para el desarrollo del cáncer de mama, por lo que convendría profundizar en su caracterización.

En cuanto a las alteraciones específicas de cada clase, no encontramos ninguna aberración genómica específica de una única clase de cáncer de mama, aunque sí que describimos diferencias significativas en las comparaciones entre dos clases tumorales (Tabla 6). Algunas de estas diferencias en nuestro estudio por cCGH (Tabla 4), como la -5q11-q23 en tumores *BRCA1* y la +3q11-q23 en tumores *BRCA2*, coincidieron con los cambios genómicos específicos asociados previamente con alguna clase (van Beers et al., 2005). Si habláramos de aberraciones frecuentes—que no específicas—de cada una de las clases de cáncer de mama en nuestro estudio por aCGH, los tumores *BRCA1* se caracterizaron por una mayor frecuencia de ganancias en 3q y 8q21.3-qtel y de pérdidas de 4q32.3-qtel y regiones diversas del brazo 5q (Figura 19). Algunas de estas aberraciones se han citado como discriminativas en las comparaciones de clases tumorales (Jonsson *et al.*, 2005; Tirkkonen *et al.*, 1997; van Beers *et al.*, 2005). Los tumores *BRCA2* presentaron ganancias frecuentes de 8q12.3-qtel y pérdidas de 11q23.1-qtel, 13q12.3-q21.33 y del cromosoma 22 (Figura 19). Es de interés resaltar que +17q22-q24 y pérdidas en el cromosoma 13 se han descrito como frecuentes en tumores *BRCA2* (Jonsson *et al.*, 2005; Tirkkonen *et al.*, 1997) y que nosotros también las hemos registrado a una alta frecuencia en tumores *BRCA2* acotándolas además a las regiones +17q23-qtel y -13q12.3-q21.33. Por su parte, los tumores BRCAX se caracterizaron por tener la menor inestabilidad genómica de todas las clases. Esto se debía probablemente al alto contenido de muestras de grado 1 en la serie BRCAX (14/31 casos), ya que tumores de bajo grado tienen un menor número de cambios genómicos (Roylance et al., 1999). Finalmente, las aberraciones genómicas frecuentes en tumores esporádicos coincidieron con las descritas previamente (Bergamaschi *et al.*, 2006; Loo *et al.*, 2004).

En lo que respecta a las amplificaciones de alto nivel, observamos que los tumores *BRCA1* presentaron una mayor tendencia a desarrollar amplificaciones que los tumores *BRCA2* y BRCAX (Figura 14) y que aquellos tumores que sufrían amplificaciones, tenían a su vez una mayor inestabilidad genómica (Figura 15). La localización genómica de las amplificaciones difirió ligeramente entre las clases tumorales (Figura 13 y Tabla 6). Por ejemplo, mientras que la amplificación de *MYC* (8q24) se registró en todos los grupos sugiriendo una región universal de amplificación, ningún tumor *BRCA1* ó *BRCA2* desarrolló amplificación de *ERBB2* (17q12-q21) en nuestros estudios ni tampoco en otros anteriores (Honrado *et al.*, 2005a; Jonsson *et al.*, 2005). De este modo, la amplificación de *ERBB2* podría ser un marcador que ayudase en la discriminación de muestras familiares en las que el análisis mutacional de los genes *BRCA* fuese aconsejable, ahorrando tiempo y esfuerzos.

1.2. EL USO DE CLASIFICADORES BASADOS EN EL PATRÓN DE ABERRACIONES GENÓMICAS: ¿ESTÁN BIEN CONSTRUIDOS?

A tenor de lo observado, el patrón de aberraciones genómicas descrito por cCGH o aCGH podría ayudar en el diagnóstico clínico ya que parecen existir algunas diferencias entre las clases tumorales (*BRCA1*, *BRCA2*, BRCAX y esporádico). Sin embargo, nuestros hallazgos también muestran discrepancias con lo observado en estudios previos, en los que se describían una serie de aberraciones discriminativas asociadas con los tumores *BRCA1* y *BRCA2* (ver Tabla 1). Estas discrepancias entre estudios se atribuyeron tradicionalmente a diferencias en el tamaño de la muestra o a los tipos de mutación de los genes *BRCA1/2* que componían cada conjunto. Sin embargo, con la presente tesis, proponemos un papel adicional según el estado del RE y el subtipo de cáncer de mama. En relación al primero, mientras que la mayoría de los estudios previos se basaron en tumores *BRCA1*/RE(-) y *BRCA2*/RE(+), nosotros hemos tenido un conjunto de muestras *BRCA1* y *BRCA2* que presentaban ambos estados del RE.

Con el objetivo de relacionar nuestros resultados con un análisis previo realizado con la misma plataforma de aCGH, incluimos nuestra serie de muestras *BRCA1*, *BRCA2* y esporádicas en un clasificador capaz de discriminar entre estas clases tumorales basándose en las aberraciones genómicas presentes en un conjunto de regiones cromosómicas (Jonsson et al., 2005). Nuestros resultados mostraron que, si bien había una tendencia a

agrupar las muestras *BRCA1* y *BRCA2* a un lado y otro del clasificador, un alto número de muestras se agrupaban erróneamente (Figura 20A). Al preguntarnos qué razón llevaba a muestras *BRCA1* a agruparse en la rama *BRCA2* y viceversa, observamos que los tumores tendían a ser agrupados en función del estado de RE. Esto podría deberse a que las regiones discriminativas de Jonsson y cols. se habían obtenido de la comparación de tumores *BRCA1*/RE(-), *BRCA2*/RE(+) y esporádicos con ambos estados, mientras que en nuestra serie todas las clases presentaron ambos estados de RE. Estos resultados sugieren un papel para el RE como marcador de los cambios genómicos que presenta un tumor. Al existir una asociación entre RE(-) y tumores *BRCA1*, la mayoría de estudios se han centrado en tumores *BRCA1* exclusivamente RE(-) no solo en caracterización genómica (Jonsson *et al.*, 2005; Tirkkonen *et al.*, 1997; van Beers *et al.*, 2005; Wessels *et al.*, 2002) sino que también en estudios de expresión (Hedenfalk et al., 2001). Sin embargo, la idea de que el estado del RE podría distorsionar los hallazgos de esta clase de estudios también ha sido mencionada (Lakhani *et al.*, 2001; Turner and Reis-Filho, 2006).

Por lo tanto, es posible que la diferente composición de estados de RE en cada una de las series sea la razón por la que se obtuvieron diferencias en las regiones cromosómicas que compusieron un clasificador basado en cCGH propuesto por nuestro grupo años atrás (Alvarez et al., 2005) con respecto a otros similares (van Beers *et al.*, 2005; Wessels *et al.*, 2002), o incluso de nuestras discrepancias obtenidas en esta tesis con Jonsson y cols. (Jonsson et al., 2005). De hecho, al homogeneizar nuestras series trabajando solo con muestras *BRCA1*/RE(-) y *BRCA2*/RE(+) el agrupamiento fue mucho más parecido al obtenido por Jonsson y cols. (Figura 20B). Si bien no ponemos en tela de juicio la validez de los clasificadores, proponemos que variables como el RE deben ser tenidas en cuenta a la hora de su construcción.

2. LA COMÚN HETEROGENEIDAD DE TODAS LAS CLASES DE CÁNCER DE MAMA FAMILIAR Y ESPORÁDICO

Uno de los hallazgos más interesantes y quizás el más importante en esta tesis ha sido descubrir que los tumores familiares *BRCA1* y *BRCA2* no representaban un conjunto homogéneo como se había venido considerando hasta la fecha. La heterogeneidad de los tumores esporádicos era conocida y en los tumores BRCAX era de esperar, pero nuestros estudios han sido los primeros en describir también la heterogeneidad de los tumores *BRCA1/2*. En las próximas páginas, centraremos la atención en la mayor importancia del estado del RE y del subtipo de cáncer de mama en el patrón de aberraciones genómicas que el de presentar mutación en *BRCA1/2*.

2.1. EL RECEPTOR DE ESTRÓGENOS ES UN MARCADOR DE LOS CAMBIOS GENÓMICOS PRESENTES EN UN TUMOR

El papel del RE como uno de los marcadores más importantes del cáncer de mama esporádico se ha visto en estudios de expresión (Perou et al., 2000) y de aCGH (Bergamaschi *et al.*, 2006; Fridlyand *et al.*, 2006; Loo *et al.*, 2004), en los que los perfiles de expresión y los de cambios genómicos difirieron claramente entre tumores RE(-) y tumores RE(+). En cuanto a los tumores familiares, como hemos mencionado anteriormente, la mayoría de estudios realizados se han centrado en grupos homogéneos de tumores *BRCA1*/RE(-) y *BRCA2*/RE(+). Sin embargo, hemos descrito que el patrón de aberraciones genómicas de un tumor *BRCA1*/RE(-) no es igual que el de un tumor *BRCA1*/RE(+), señalando así que la heterogeneidad a nivel del RE también está presente en los tumores de mama familiar.

En nuestro análisis, los tumores RE(-) siempre presentaron una mayor inestabilidad genómica que los tumores RE(+) tanto al agrupar todos los tumores (*BRCA1/2*/X y esporádicos) en función de su estado del RE, como al subdividirlos por la clase de cáncer de mama (Figura 21). El patrón de aberraciones genómicas también resultó muy diferente entre los tumores RE(-) y RE(+) (Figura 22A). Un conjunto de aberraciones genómicas, más frecuentes en los tumores RE(-), discriminó de manera significativa ambas clases de tumores: -3p25.3-p21.3, -3p21.1-p14.2, +3q25.1-q26.1, -4q31.2-qtel, -5q, +10p14-p12.3, -12q14-q15 y -12q23.1-q23.31 (Figura 22B). La mayoría de estas aberraciones coincidían

con las descritas previamente en estudios de aCGH de cáncer de mama esporádico (Bergamaschi *et al.*, 2006; Fridlyand *et al.*, 2006; Loo *et al.*, 2004) y algunas de ellas se incluían además entre las regiones discriminativas de Jonsson y cols. (Jonsson et al., 2005). De hecho, pérdidas genómicas en diversos loci de 4q y 5q se habían descrito como frecuentes en tumores *BRCA1* pero también en muestras *BRCA2* y esporádicas con negatividad para RE (Johannsdottir *et al.*, 2004; Johannsdottir *et al.*, 2006). Por su parte, los tumores RE(+) mostraron menor inestabilidad genómica y alteraciones frecuentes en el cromosoma 16 (+16p y -16q), aberraciones clásicas de tumores RE(+) y de bajo grado (Loo *et al.*, 2004; Roylance *et al.*, 1999; Roylance *et al.*, 2006). Además, los tumores RE(-) tuvieron un mayor número de amplificaciones de alto nivel que los tumores RE(+) diferenciándose en las regiones afectadas y en su frecuencia (ver Resultados 2.4). En general, los diferentes patrones genómicos en tumores RE(-) y tumores RE(+) se mantuvieron al subdividir los grupos en tumores *BRCA1*/*2*/X y esporádicos (Figura S 1), lo cual enfatiza el papel del estado del RE como marcador de los cambios genómicos presentes en un tumor, mucho más importante que la clase tumoral. En este sentido, las comparaciones entre clases tumorales con un mismo estado de RE podrían dilucidar aberraciones específicamente asociadas a una clase tumoral, aunque para ello es necesario un mayor número de muestras.

Llegados a este punto, querríamos hacer un inciso para debatir sobre la razón biológica de la ausencia de expresión de RE en los tumores RE(-). A tenor de nuestros resultados, este fenómeno se debe a un mecanismo independiente al de pérdidas genómicas del locus *ESR1*, dado que los tumores RE(+) presentaron una mayor frecuencia de deleción de esa región que los tumores RE(-) (Figura 22A). Otro mecanismo de pérdida de expresión de RE podría ser la hipermetilación del promotor de *ESR1* que, si bien no la hemos estudiado, se ha descrito en la literatura en un pequeño grupo de tumores RE(-) acompañada de otros procesos paralelos (desacetilación de histonas, relaciones entre promotores y reguladores de la transcripción, etc.). No obstante, los autores también describieron hipermetilación de *ESR1* en tumores RE(+), explicando así el diferente porcentaje de células positivas para RE en esta clase de tumores (Parrella et al., 2004). Este debate adquiere mayor complejidad al combinarlo con los diferentes orígenes celulares que un cáncer de mama podría presentar y que veremos más adelante (Dontu et al., 2004).

En resumen, nuestros hallazgos sugieren un papel crítico del estado del RE como marcador de los cambios genómicos presentes en un tumor. Los patrones de aberraciones genómicas entre cáncer de mama esporádico y familiar son bastante similares y las diferencias parecen determinarse principalmente por el estado del RE en lugar de la mutación *BRCA* como se aceptaba tradicionalmente. La negatividad para RE podría permitir o favorecer la aparición de aberraciones genómicas diferentes a las presentes en tumores que expresan RE y estas regiones podrían contener genes interesantes que determinarían la mayor agresividad de los tumores RE(-). Sin embargo, también hemos dilucidado con esta tesis que existe heterogeneidad más allá de los grupos definidos por la expresión del RE: una heterogeneidad dependiente del subtipo molecular del cáncer de mama a la que dedicaremos el siguiente apartado.

2.2. EL CÁNCER DE MAMA FAMILIAR TAMBIÉN PRESENTA DIFERENTES SUBTIPOS MOLECULARES QUE LLEVAN ASOCIADOS DISTINTOS PATRONES DE ABERRACIONES GENÓMICAS

Vista la heterogeneidad del RE en cada una de las clases de cáncer de mama, nos preguntamos si los distintos subtipos moleculares descritos en tumores esporádicos se mostraban a su vez en los tumores familiares.

2.2.1. El cáncer de mama familiar se subdivide en subtipos IHQ

Al basarnos en un estudio que definía la existencia de varios subtipos IHQ en tumores BRCAX y esporádicos desarrollado por nuestro grupo (Honrado et al., 2007), vimos resultados similares considerando todas las clases de cáncer de mama familiar. Registramos cinco subtipos IHQ (Figura 23) similares a los encontrados en tumores esporádicos por estudios de expresión: subtipo basal, ERBB2, luminal A y luminal B (Perou *et al.*, 2000; Sorlie *et al.*, 2001). Aunque en nuestra serie no identificamos el subtipo similar a tejido normal, registrado en los estudios previos, describimos un quinto subtipo con características intermedias entre los subtipos luminal A y luminal B, al cual nombramos como “no-clasificado”. Dado su alto grado y sobreexpresión de marcadores de proliferación (ciclinas, Ki-67, etc.), este subtipo podría ser más agresivo que el subtipo luminal A y, de este modo, asemejarse a los subtipos luminal C ó luminal 3 propuestos con anterioridad (Sorlie *et al.*, 2001; Sotiriou *et al.*, 2003). La importancia de estos hallazgos

radica en la común existencia de diferentes subtipos de cáncer de mama en tumores esporádicos y familiares.

Sin embargo, la proporción de estos subtipos en tumores familiares no es la misma que en tumores esporádicos (Sorlie et al., 2001) o muestras BRCAX únicamente (Honrado *et al.*, 2007; Oldenburg *et al.*, 2006). En nuestro análisis, el 26% de tumores familiares eran de subtipo basal comparado al 15% registrado en esporádico y BRCAX (Oldenburg *et al.*, 2006; Sorlie *et al.*, 2001) (Tabla 7). Esta diferencia puede radicar en la presencia de tumores *BRCA1* en nuestra serie, los cuales son habitualmente de fenotipo basal (Sorlie *et al.*, 2003; Tischkowitz and Foulkes, 2006; Turner and Reis-Filho, 2006). En nuestra serie, el 61% de los tumores *BRCA1* representaron una proporción importante de los tumores basales familiares. El 19% de los tumores *BRCA2* y el 7% de los casos BRCAX también presentaron fenotipo basal (Tabla 7). Todos los tumores BRCAX habían sido analizados previamente para hipermetilación del promotor y pérdida de heterocigosidad de *BRCA1* (datos no mostrados), destacando que aquellas muestras BRCAX con fenotipo basal mostraron inactivación bialélica de *BRCA1* (Honrado et al., 2007). Por lo que un importante papel de *BRCA1* podría existir en el desarrollo del subtipo basal del que hablaremos más adelante. Por su parte, el subtipo ERBB2 se compuso exclusivamente de tumores BRCAX (~14% de los mismos, Tabla 7), lo que coincide con nuestros resultados previos en los que no había amplificación de *ERBB2* y con aquellos que citan una baja incidencia de la sobreexpresion de este gen en tumores *BRCA1/2* (Adem *et al.*, 2004; Grushko *et al.*, 2002; Lakhani *et al.*, 2002; Palacios *et al.*, 2003). También se encontró una asociación significativa entre tumores BRCAX (45%) y el subtipo luminal A, en concordancia con lo ya descrito (Honrado *et al.*, 2007; Oldenburg *et al.*, 2006). Por último, los tumores *BRCA2* y los *BRCA1* que no eran basales mostraron principalmente un subtipo luminal B (37% y 22%, respectivamente); si bien los tumores *BRCA2* también representaron una importante proporción de las muestras “no-clasificadas” (Tabla 7). Por el contrario, Sorlie y cols. asociaron los tumores *BRCA2* al subtipo luminal A (Sorlie et al., 2003). Esta discrepancia podría deberse al número de muestras, ya que Sorlie y cols. solo utilizaron dos casos mientras que nosotros tuvimos una serie mayor de 16 muestras.

2.2.2. Cada subtipo IHQ se asocia a un patrón de aberraciones genómicas

Hemos confirmado en los subtipos familiares lo que estudios recientes en los cinco subtipos esporádicos han descrito (Bergamaschi *et al.*, 2006; Chin *et al.*, 2006): diferentes patrones genómicos se asocian a cada subtipo de cáncer de mama. Los tumores basales y los ERBB2 (en familiares) o luminal A (en esporádicos) mostraron la mayor y menor inestabilidad genómica, respectivamente. La discrepancia sobre el subtipo menos inestable puede deberse al bajo número de tumores ERBB2 en nuestro estudio, por lo que no descartamos una menor inestabilidad de los familiares luminal A.

El patrón de aberraciones genómicas difirió considerablemente entre subtipos (Figura 25). Los tumores basales mostaron inestabilidad en muchas regiones cromosómicas (Tabla 13), algunas de las cuales se asociaban significativamente (-3p25, -4p, -4q22-q35.1, -5q). La asociación de los tumores *BRCA1* al subtipo basal explicaría las similitudes entre los patrones genómicos clásicamente asociados a *BRCA1* y los tumores basales, lo que propone un papel más importante al subtipo tumoral que a la mutación. Los tumores luminal A presentaban frecuentemente +1q, +16p, -11q23 y -16q; mientras que los tumores luminal B mostraron aberraciones más diversas (Tabla 13). Indistintamente, estos patrones fueron muy similares a los descritos en los subtipos esporádicos (Figura 33) (Bergamaschi *et al.*, 2006; Chin *et al.*, 2006).

En cuanto a las amplificaciones de alto nivel, el subtipo luminal B es el que desarrolla una mayor cantidad de estas aberraciones igual que en tumores esporádicos (Bergamaschi *et al.*, 2006; Chin *et al.*, 2006). La localización de estas amplificaciones también difiere: como cabía esperar, los tumores ERBB2 tenían una frecuente amp17q12 (locus *ERBB2*). Por su parte, el subtipo luminal B desarrolló amplificaciones recurrentes en 8p11-p12, 8q24, 11q13.3-q13.4, 17q25 y 20q13 (Figura 26B); mientras que los luminal A solo compartían la frecuente amp11q13 (locus de *CCND1*). Esta aberración común en tumores luminales podría explicar la sobreexpresión de CCND1 en los mismos (Figura 23) y está en concordancia con estudios que muestran una correlación inversa entre estos fenómenos y tumores basales (Elsheikh *et al.*, 2007; Reis-Filho *et al.*, 2006). Por último, los tumores basales amplificaron frecuentemente 8q24, 12p13 y 13q34 (Figura 26B). Estudiamos la amp13q34, acotándola a una región mínima de 1’6Mb, y la vimos asociada significativamente a una sobreexpresión de TFDP1, factor de transcripción que favorece la expresión de ciclina E y otros genes del ciclo celular, si bien la amplificación no fue su

único mecanismo de sobreexpresión. La mayoría de estas regiones de amplificación fueron similares a las descritas en los subtipos esporádicos (Bergamaschi *et al.*, 2006; Chin *et al.*, 2006). La existencia común de estas amplificaciones podría representar dianas terapéuticas como se ha establecido para *ERBB2* y trastuzumab.

Tabla 13. Resumen de la proporción y características genómicas de los subtipos de tumores familiares: basal, ERBB2, luminal A y luminal B

Subtipo IHQ	Clase de cáncer de mama familiar (%)			Inestabilidad genómica	Ganancias recurrentes	Pérdidas recurrentes	Amplificaciones de alto nivel	Regiones de amplificación
	BRCA1	BRCA2	BRCAX					
Basal	68'75	18'75	12'50	Alta	1q, 3q, 8q, 17q	3p, 4p, 4q, 5q, 8p, 10q, 11q, 12q, 13q, 14q, 15q, 16q, 22q	Media	6p22, 8q24, 13q34
ERBB2	0	0	100	Baja	1q, 17q, Xp	3p, 4q, 8p, 11q	Media	8q24, 17q12-q21
Luminal A	6'25	12'50	81'25	Baja	1q, 16p	11q, 16q	Baja	11q13
Luminal B	23'53	35'30	41'17	Media	8q, 16p, 20q	8p, 11q, 14q, 16q, 22q	Alta	8p11-12, 11q13, 20q13

Las columnas muestran la distribución de cada subtipo IHQ en cada una de las clases tumorales familiares; el nivel de inestabilidad genómica (ver Figura 24 para mayor detalle), con las ganancias y pérdidas recurrentes (Figura 25); y la cantidad y regiones de amplificación de alto nivel (Figura 26).

En conclusión, distintos subtipos existen en tumores familiares y esporádicos aunque con una asociación de *BRCA1* con basales y de BRCAX con luminal A que merece especial atención. Los diversos rasgos genómicos de estos subtipos concordaron también, siendo los tumores basales los más inestables aunque el subtipo luminal B desarrolló más amplificaciones de alto nivel. Estos hallazgos apoyan la existencia de diferentes rutas genéticas de evolución tumoral, comunes a tumores esporádicos y familiares, que subyacen en el origen de los distintos subtipos de cáncer de mama y que podría explicar sus diversos patrones moleculares. Creemos también que el perfil de expresión, la manifestación clínica y la respuesta al tratamiento serán diferentes en los subtipos familiares, como lo son en los esporádicos (Carey *et al.*, 2006; Hu *et al.*, 2006; Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003). Con todo lo anterior, los subtipos de cáncer de mama deberían tratarse como entidades diferentes para establecer correctas comparaciones en pos de caracterizar apropiadamente los posibles rasgos asociados a mutaciones en *BRCA1/2*, como ya se ha propuesto con tumores basales *BRCA1* y basales esporádicos (Turner and Reis-Filho, 2006; Vincent-Salomon *et al.*, 2007; Yehiely *et al.*, 2006).

3. PROPUESTA DE UN MODELO INTEGRADOR DEL ORIGEN Y DESARROLLO DE LOS SUBTIPOS DE CÁNCER DE MAMA FAMILIAR Y ESPORÁDICO

El tejido mamario se compone de una población de células indiferenciadas compuestas de células troncales, capaces de renovarse y de diferenciarse en linajes celulares varios, y de progenitores celulares, descendientes directos de las células troncales con capacidad proliferativa y cuya diferenciación está comprometida. Al final de estos linajes celulares, se encuentra la población de células mamarias diferenciadas: mioepiteliales, ductales y alveolares.

Por otro lado, se supone que el cáncer de mama se inicia por la carcinogénesis en un núcleo de células. Existen dos modelos de carcinogénesis: el modelo estocástico o de selección clonal, en el que la malignización puede darse por múltiples mutaciones en una célula aleatoria y cuyos clones se someten a una posterior selección; y el modelo jerárquico o de célula troncal cancerígena, en la que la malignización se daría en células troncales y/o progenitoras por desregulación de las rutas de renovación (Wicha et al., 2006). En este sentido, la reciente identificación de células humanas iniciadoras del cáncer de mama apoya la capacidad carcinogénica única de un conjunto de células bajo un apropiado nicho celular (Al-Hajj et al., 2003). De acuerdo al modelo jerárquico, el tipo de evento carcinogénico y la célula afectada son las causas inherentes de la heterogeneidad del cáncer de mama. De este modo, la carcinogénesis en células troncales o progenitoras RE(-) serían el origen de los subtipos basales, ERBB2 y luminal B, mientras que en células progenitoras RE(+) serían la raíz biológica del subtipo luminal A (Behbod and Rosen, 2005; Dontu *et al.*, 2004)

¿Cómo encaja en este cuadro el cáncer de mama familiar y la heterogeneidad que hemos registrado en él en nuestros estudios? De acuerdo a nuestros resultados, proponemos un modelo integrador para explicar el origen y desarrollo de los diferentes subtipos de cáncer de mama familiar y esporádico. En este modelo (Figura 32), defendemos un origen jerárquico de los subtipos tumorales en el que las células troncales y progenitoras RE(-) podrían verse afectadas por numerosos eventos carcinogénicos de los que destacarían: a) el silenciamiento de *BRCA1*, al que creemos regulador de la

diferenciación de las células troncales y que, al no ser funcional, produciría la indiferenciación celular característica de los tumores basales; b) la amplificación y/o sobreexpresión de ERBB2 como el evento carcinogénico original de los tumores ERBB2; c) importantes ventajas proliferativas producto de la amplificación y/o sobreexpresión de proto-oncogenes originando tumores luminal B. Finalmente, la carcinogénesis en células progenitoras RE(+) generarían los tumores luminal A. Asimismo, la caracterización genómica de estos subtipos nos permite describir aberraciones comunes: 1) a todos los subtipos tumorales, que podrían representar desequilibrios genómicos iniciales y necesarios para el desarrollo de cáncer de mama; 2) a todos los subtipos procedentes de células iniciadoras RE(-), que podrían indicar el origen celular común o una selección; y 3) a tumores RE(+) con diferentes células iniciadoras, que podrían indicar una selección clonal combinando así ambos modelos carcinogénicos.

3.1. LA RUTA DEL SUBTIPO BASAL: LA ESTRECHA RELACIÓN DE *BRCA1* Y LAS CÉLULAS TRONCALES CANCERÍGENAS DE MAMA

Esta tesis nos ha ayudado a entender que la estrecha similitud que hay en numerosos marcadores IHQ, de expresión y genómicos entre los tumores *BRCA1* tratados como un grupo homogéneo y los tumores basales se debe a que la mayoría de los tumores *BRCA1* presentan un fenotipo basal. Sin embargo, este subtipo también se registra entre los tumores *BRCA2*, BRCAX y esporádicos con una frecuencia ~15% (Honrado *et al.*, 2007; Melchor *et al.*, 2007b; Sorlie *et al.*, 2001). Como mencionamos con anterioridad, hemos visto que aquellos tumores BRCAX con fenotipo basal presentaron doble inactivación de *BRCA1* (Honrado *et al.*, 2007); mientras que en esporádicos existe una frecuente hipermetilación del promotor de *BRCA1* en carcinomas metaplásicos (un grupo dentro de los carcinomas basales) o bien un silenciamiento de *BRCA1* por mecanismos diferentes como la alta expresión de ID4, un represor de BRCA1 (Turner *et al.*, 2007). Estos hallazgos apuntan a un papel crucial de *BRCA1* en la carcinogénesis de los tumores basales, pudiendo tratarse de un regulador necesario para que la célula troncal derive a una diferenciación luminal de modo que, en su ausencia, permanecerían en el tejido los rasgos indiferenciados característicos del fenotipo basal (Foulkes, 2004).

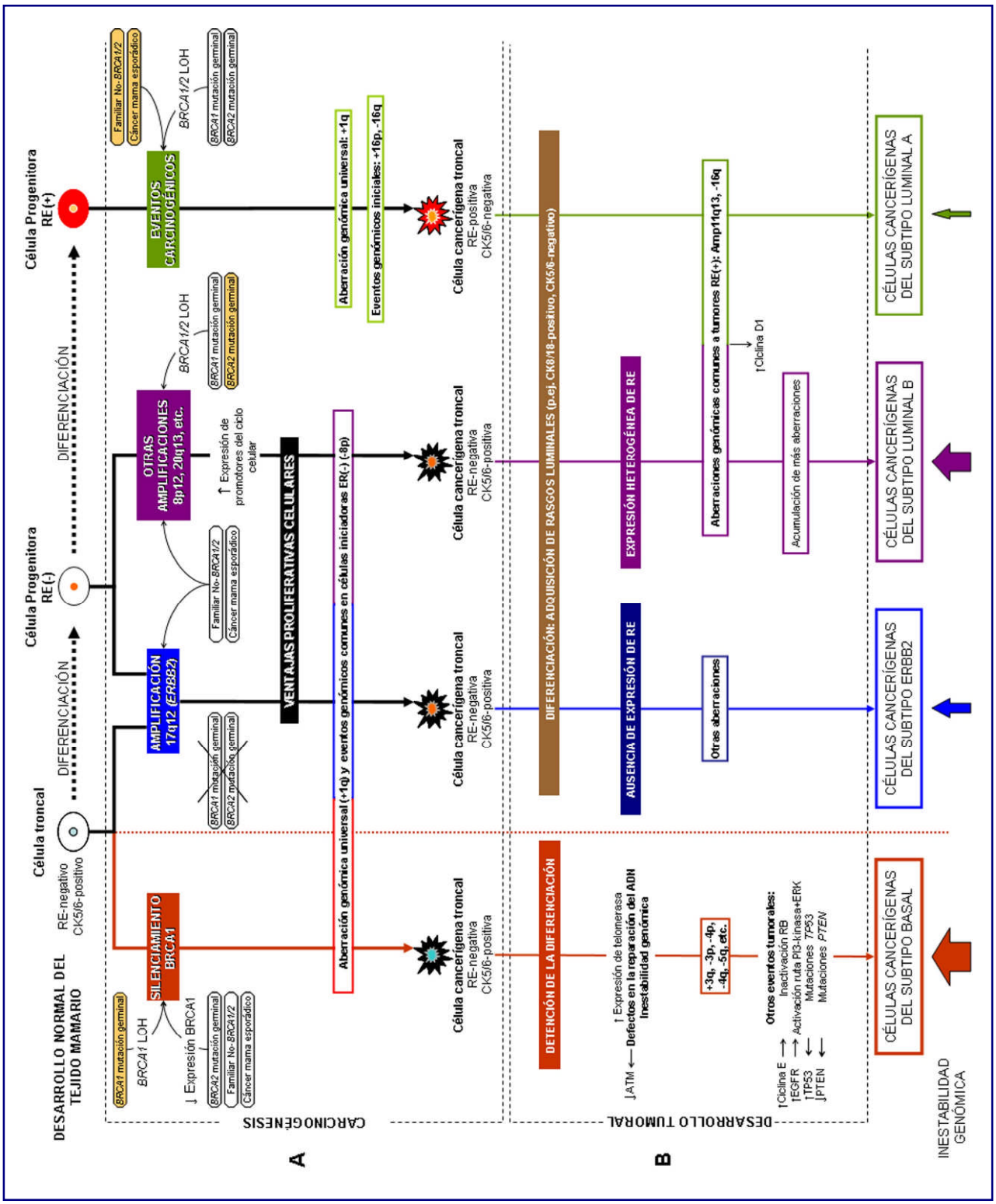
Por lo tanto, el evento carcinogénico más importante en el origen de los carcinomas basales sería la pérdida del alelo normal y/o el silenciamiento de *BRCA1* en las células

troncales normales de pacientes portadores de mutación en *BRCA1* o de aquellos no portadores, respectivamente (Figura 32A). Acto seguido, durante el desarrollo tumoral sucederían eventos tales como (Yehiely *et al.*, 2006) (Figura 32B): a) la detención de los procesos de diferenciación luminal, lo que daría lugar al fenotipo indiferenciado de los carcinomas basales; b) el incremento de actividad de la telomerasa, al no haber BRCA1 que reprima la expresión de la misma; c) defectos en la reparación del ADN generando inactivación o pérdida de ATM (Tommiska *et al.*, 2007) así como un incremento en la inestabilidad genómica, posible causa del mayor número de aberraciones genómicas de los carcinomas basales esporádicos y familiares (Figura 33); d) la inactivación de la ruta RB por medio de la sobreexpresión de CCNE; e) la activación de la ruta PI3-Kinasa al sobreexpresarse EGFR; f) mutaciones de *TP53* que le llevan a sobreexpresarse; y g) aberraciones genómicas de *PTEN* (10q23.31) que llevan a la pérdida de expresión del mismo (Saal *et al.*, 2008). Los análisis IHQ y de expresión corroboran los niveles de estos marcadores como característicos de carcinomas basales (Livasy *et al.*, 2006; Nielsen *et al.*, 2004) y, por ende, de la mayoría de los tumores *BRCA1* (Lakhani *et al.*, 2002; Palacios *et al.*, 2005b; Palacios *et al.*, 2003).

3.2. LA RUTA DEL SUBTIPO ERBB2: LA INCOMPATIBILIDAD DE LA AMPLIFICACIÓN Y/O SOBREEXPRESIÓN DE ERBB2 EN TUMORES DE PORTADORES DE MUTACIÓN *BRCA1/2*

El subtipo ERBB2 se encuentra presente en un 15-20% de tumores esporádicos y BRCAX (Honrado *et al.*, 2007) pero, a lo largo de nuestros análisis, hemos descrito siempre la ausencia de amplificación/sobreexpresión de *ERBB2* en los tumores portadores de mutación *BRCA1/2*, algo que en la literatura ya se registra con una incidencia baja o inexistente (Adem *et al.*, 2004; Grushko *et al.*, 2002; Lakhani *et al.*, 2002; Palacios *et al.*, 2003). ¿Por qué los tumores *BRCA1/2* no amplifican y/o sobreexpresan *ERBB2*? Hace una década, se propuso que los portadores de mutación *BRCA1* no amplificaban *ERBB2* al tener como evento carcinogénico la pérdida alélica de *BRCA1* y, por ende, de *ERBB2* que está situado muy cerca en el genoma (Johannsson *et al.*, 1997). Sin embargo, esta teoría no explicaría la ausencia de sobreexpresión de ERBB2 en los tumores *BRCA2*. Una posible alternativa es que aquellas células cancerígenas que posean mutaciones en los genes *BRCA1/2* y que desarrollen una sobreexpresión de ERBB2 no tengan ventajas selectivas y, por lo tanto, no proliferarían. Es posible que los defectos en la reparación del ADN no sean

compatibles con el estrés de señales proliferativas ofrecido por la tirosina-quinasa ERBB2 y, por lo tanto, las células tumorales con sobreexpresión de ERBB2 que sufran importantes cambios genómicos no sobrevivan. La baja inestabilidad genómica registrada en los tumores ERBB2 esporádicos y familiares podrían apoyar esta idea (Figura 33).



De todos los eventos carcinogénicos, el más importante para desarrollar el subtipo ERBB2 sería la amplificación y/o sobreexpresión temprana de ERBB2 en una célula troncal o progenitora ER(-) (Figura 32A) tal y como se ha postulado en el modelo jerárquico (Behbod and Rosen, 2005). A continuación, dado que *BRCA1* no está alterado, la célula troncal cancerígena sería capaz de diferenciarse con la expresión de citoqueratinas luminales, si bien no adquiriría la expresión de RE (Figura 32B).

3.3. LA RUTA DEL SUBTIPO LUMINAL B: ADQUISICIÓN DE VENTAJAS PROLIFERATIVAS

El subtipo luminal B se presenta en un ~15% de todas las clases de cáncer de mama y está caracterizado por un alto grado, expresión variable de receptores hormonales, expresión de citoqueratinas luminales y sobreexpresión de reguladores del ciclo (*CCNE*) o del crecimiento celular (*TOPIIA*) (Sorlie *et al.*, 2001). Desde el punto de vista genómico, es el subtipo que más amplificaciones de alto nivel presenta (Bergamaschi *et al.*, 2006; Chin *et al.*, 2006; Melchor *et al.*, 2007b). Otro rasgo característico de este fenotipo es su peor pronóstico con respecto a los tumores luminal A (Sorlie *et al.*, 2001). El modelo jerárquico propone que los tumores luminal B derivan de células progenitoras RE(-) capaces de diferenciarse y desarrollar una expresión variable de RE (Behbod and Rosen, 2005; Dontu *et al.*, 2004) (Figura 32). Esto explicaría el porcentaje de pacientes que, aún desarrollando tumores RE(+) y siendo tratados con terapias hormonales como el tamoxifeno, tienen reapariciones del tumor, dado que las células cancerígenas troncales son RE(-). Asimismo, esto también explicaría la mayor tasa de recidiva de los luminales B.

Figura 32. Modelo integrador de la carcinogénesis y desarrollo tumoral de las células troncales mamarias. A, carcinogénesis y producción de células cancerígenas troncales. Diversos eventos carcinogénicos suceden en cada una de las células troncales o progenitoras normales que componen el tejido mamario. Las clases de pacientes se muestran en recuadros anexos al evento carcinogénico principal. Los recuadros amarillos señalan la ruta más frecuente que siguen los tumores originados en esos pacientes. Los recuadros tachados de los portadores de mutación BRCA1/2 en la ruta ERBB2 señalan la posible incapacidad de sus células de derivar por esa ruta. Se muestran también las aberraciones genómicas universales en cada ruta así como aquellas que podrían ser "eventos iniciales" en la carcinogénesis de células troncales o progenitoras RE(-) y células progenitoras RE(+). Al final, se muestran las células cancerígenas troncales, en las que un núcleo naranja señalaría una capacidad variable de diferenciación. B, desarrollo tumoral y producción de células cancerígenas de cada uno de los subtipos tumorales. Se muestra el conjunto de fenómenos que ocurren durante el desarrollo del tumor tales como los procesos de diferenciación, la adquisición de aberraciones genómicas y de otros eventos tumorogénicos. La inestabilidad genómica se muestra debajo de cada una de los subtipos tumorales con una flecha cuyo grosor representa el grado de inestabilidad.

¿Pero qué evento carcinogénico determina un subtipo luminal B? Podemos suponer que, del mismo modo que los tumores ERBB2 dependen de una amplificación y/o sobreexpresión temprana de ERBB2, los tumores luminal B dependerán de una amplificación y/o sobreexpresión temprana de promotores del ciclo celular (Figura 32A). Amplificaciones iniciales podrían darse en 8p11-p12, 8q21-q24 o 20q13, dada su elevada frecuencia en el subtipo luminal B, lo que llevaría a una ventaja proliferativa en una célula troncal cancerígena capaz de diferenciarse adquiriendo rasgos luminales y expresión de RE. Es posible que, durante el desarrollo tumoral, estos clones desarrollen nuevas amplificaciones ya que tumores con la inestabilidad necesaria para originar una amplificación son proclives a desarrollar múltiples amplificaciones adicionales (Al-Kuraya *et al.*, 2004; Melchor *et al.*, 2005).

¿Y qué diferencia hay entre los portadores de mutación *BRCA1* que desarrollan un subtipo basal o uno luminal B? ¿Es posible que no pierdan el alelo normal *BRCA1*? Contestamos a esta pregunta analizando la LOH del gen en aquellos tumores *BRCA1* con un subtipo diferente al basal y observamos que también presentaban pérdida alélica (datos no mostrados) por lo que estos tumores tienen ambas copias del gen inactivadas. Proponemos otras posibles alternativas son: 1) la pérdida alélica de *BRCA1* se da en una célula troncal RE(-) en los tumores basales y en una célula progenitora RE(-) en los tumores luminal B; 2) el tipo de mutación de *BRCA1*, dado que algunas mutaciones dan lugar a proteinas aberrantes o truncadas que no disparan el sistema “non-sense mediated decay” (NMD) (Anczukow *et al.*, 2008; Buisson *et al.*, 2006; Perrin-Vidoz *et al.*, 2002). De hecho, en nuestra serie, la mutación 185delAG de *BRCA1*, que produce un ARNm truncado que evita el sistema NMD (Buisson *et al.*, 2006), se halla preferentemente en los tumores *BRCA1* que desarrollan un subtipo basal (12/13 portadores de mutación 185delAG de *BRCA1*) (datos no mostrados). Sin embargo, son necesarias más muestras para confirmar alguna de estas teorías.

3.4. LA RUTA DEL SUBTIPO LUMINAL A: LA CARCINOGÉNESIS EN UNA CÉLULA PROGENITORA RE(+)

Este subtipo está presente en la mayoría (~40%) de los tumores esporádicos y BRCAX (Carey *et al.*, 2006; Honrado *et al.*, 2007; Hu *et al.*, 2006; Sorlie *et al.*, 2001) y en una minoría (~15%) de *BRCA1* y *BRCA2* (Melchor *et al.*, 2007b). Es el subtipo menos

agresivo (Sorlie *et al.*, 2001) y con menor inestabilidad genómica de todos (Figura 33). Su carcinogénesis posiblemente tiene lugar en células progenitoras RE(+) originando células troncales cancerígenas RE(+), capaces de diferenciarse y susceptibles a terapias hormonales (Dontu *et al.*, 2004) (Figura 32A). Dado que la esperanza de vida de una célula troncal normal es superior al de las progenitoras RE(+), aquéllas son susceptibles de acumular múltiples mutaciones. De este modo, la pérdida alélica de *BRCA1/2* en una célula portadora de mutación sería superior en las células troncales que en las progenitoras y esto explicaría la baja frecuencia del subtipo luminal A en los tumores *BRCA1/2*. Sin embargo, cuando la pérdida alélica se da en una célula progenitora RE(+), se originarían tumores luminal A. Desconocemos qué eventos carcinogénicos pueden ser los más importantes en esta ruta pero lo crucial es que la célula que se transforma es una progenitora RE(+). En el desarrollo tumoral, habría una mayor diferenciación luminal independientemente del estado de *BRCA1*, dado que las células progenitoras no están bajo su influencia. Asimismo, la baja inestabilidad genómica podría resultar de la autosuficiencia generada por las señales de estrógeno reconocidas por el RE y en la que cualquier desequilibrio genómico puede no ofrecer ventajas proliferativas.

3.5. LAS ABERRACIONES GENÓMICAS PUEDEN APOYAR UNA SELECCIÓN CLONAL

Las aberraciones genómicas se han utilizado para dilucidar rutas genéticas de evoluciones tumorales como, por ejemplo, la discriminación de dos rutas diferentes de los tumores de grado I (menos inestables) y los de grado III (más inestables) por lo que un tumor de grado I no evoluciona a grado III (Roylance *et al.*, 1999; Simpson *et al.*, 2005). Si aplicamos nuestros conocimientos del patrón de aberraciones genómicas descrito en los diferentes subtipos, podemos ver una serie de cambios genómicos comunes entre algunos de estos subtipos que podrían apoyar el hecho de una selección clonal ante determinadas variables del nicho celular (Figura 33).

No cabe duda de que las similitudes entre los patrones de aberraciones genómicas de los subtipos esporádicos y familiares apoyan un origen y unas rutas comunes entre ambas categorías de cáncer de mama (Figura 33). Si nos fijamos en aquellas aberraciones presentes en >50% en todos los subtipos, nos encontraríamos con +1q, aberración ésta que ha sido descrita por cCGH como evento temprano en el cáncer de mama (Buerger *et al.*,

2001; Tirkkonen *et al.*, 1997). Recientemente, la caracterización por aCGH ha descrito regiones mínimas de ganancia en 1q que contenían importantes genes relacionados con proliferación, transcripción y tráfico celular (Orsetti *et al.*, 2006). Por lo tanto, +1q puede resultar un evento necesario para la desregulación de estas rutas y, de este modo, favorecer la aparición del cáncer de mama (Figura 32A).

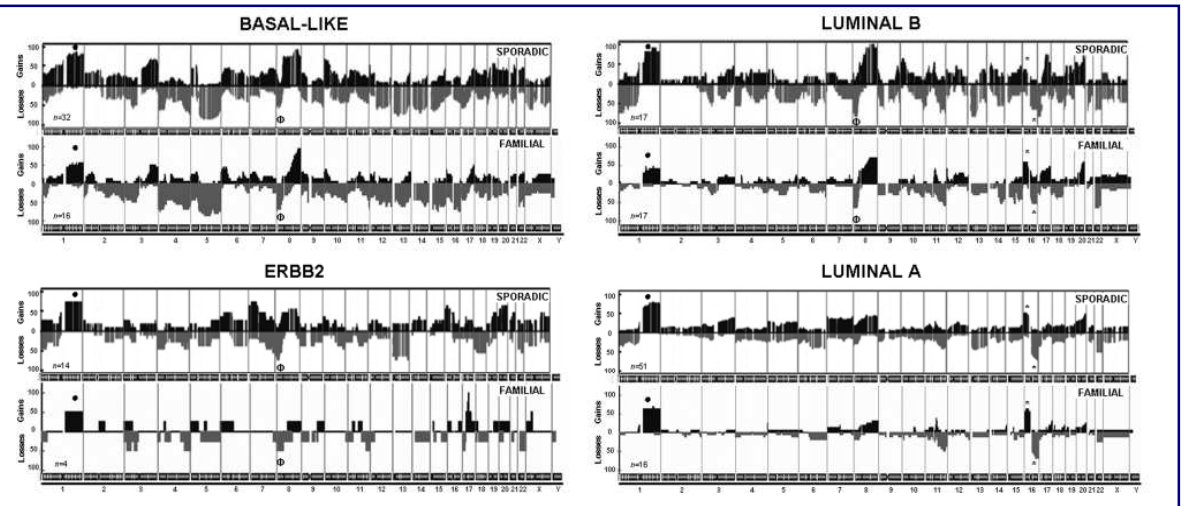


Figura 33. Comparación de los perfiles de aberraciones genómicas de los subtipos de cáncer de mama esporádico y familiar. Los perfiles proceden de dos estudios previos (Chin *et al.*, 2006; Melchor *et al.*, 2007b). Las regiones señaladas en (●) representan regiones comunes a todos los subtipos de cáncer de mama, en (Φ) aquellas comúnmente alteradas en tumores procedentes de células troncales cancerígenas RE(-), y en (*) aquellas comunes a tumores con expresión de RE.

Si prestamos atención a los tumores que proceden de células troncales cancerígenas RE(-) (basales, ERBB2 y luminal B) y observamos aquellas aberraciones genómicas que les caractericen por no estar presentes en una igual frecuencia en los tumores procedentes de células troncales cancerígenas RE(+) (tumores luminal A), registramos la pérdida de 8p (Figura 33). Esta región ha sido objeto de múltiples estudios en busca de un gen supresor tumoral pero la común existencia en estos tres subtipos sugiere que aquellos clones de células cancerígenas RE(-) con -8p pueden tener una mayor ventaja. Es decir, que la aberración genómica puede ser importante para cualquiera de los procesos carcinogénicos que se producen en estas entidades celulares.

Un dato a resaltar de estos perfiles es la heterogeneidad en el patrón genómico que existen dentro de los tumores RE(-) (basales y ERBB2) y RE(+) (luminal A y B). Sin embargo, aunque hay diferencias, existen cambios genómicos especialmente frecuentes en tumores RE(+) como +16p, -16q y la amp11q13 (locus de *CCND1*) (Figura 33). La pérdida de 16q parece ser un evento inicial en carcinomas lobulillares o de bajo grado, mientras

que se considera un evento tardío en los tumores de alto grado probablemente debido a una alta inestabilidad genómica (Roylance *et al.*, 1999; Roylance *et al.*, 2006). Dado que la mayoría de los tumores luminal A son de bajo grado y los luminal B de grado II ó III (Callagy *et al.*, 2003; Melchor *et al.*, 2007b; Sotiriou *et al.*, 2003), podría ser que -16q se produzca en ambos subtipos por mecanismos diferentes: siendo en tumores luminal A un evento temprano y en tumores luminal B un evento tardío producto de la inestabilidad genómica (Figura 32). Asimismo, la amp11q13 con la sobreexpresión de CCND1 se asocia con RE(+) (Elsheikh *et al.*, 2007; Reis-Filho *et al.*, 2006). En definitiva, estas aberraciones genómicas podrían ofrecer una ventaja selectiva a aquellos clones celulares con RE pero no en aquellos que no presentaran expresión de RE.

En resumen, este modelo integrador explicaría las rutas comunes entre el cáncer de mama familiar y esporádico para la carcinogénesis y el desarrollo tumoral de los diferentes subtipos tumorales. Apoyamos un modelo jerárquico en el inicio del tumor en base a las evidencias de la literatura y nuestras propuestas, y lo combinamos con un modelo de selección clonal durante el desarrollo tumoral gracias a las aberraciones genómicas registradas. Si bien la heterogeneidad es común a todas las clases de cáncer de mama, los tumores *BRCA1* tienen una tendencia a desarrollar carcinomas basales y esto puede radicar en la importancia de *BRCA1* como un regulador de la diferenciación de células troncales normales. Todavía queda discernir el papel que puede jugar *BRCA2* en este modelo y especialmente abordar una validación experimental de cada una de las rutas genéticas que originan los distintos subtipos moleculares de cáncer de mama.

CONCLUSIONES

1. La caracterización genómica del cáncer de mama familiar y esporádico ha permitido definir aberraciones genómicas comunes a todas las clases tumorales: +1q, +16p, -8p y -16q. Estas alteraciones podrían representar desequilibrios genómicos necesarios para el origen y desarrollo de los tumores de mama.

2. El cáncer de mama familiar asociado a mutaciones en *BRCA1/2* presenta mayor inestabilidad genómica que los tumores BRCAX y esporádicos, así como una tendencia a alterar un conjunto de regiones cromosómicas específicas en cada grupo.

3. Hemos demostrado una heterogeneidad en el cáncer de mama familiar que reproduce la que existe en tumores esporádicos. El estado del receptor de estrógenos (RE) es un marcador del patrón de aberraciones genómicas más importante que la mutación en los genes *BRCA*, de modo que tumores RE(-) presentan mayor inestabilidad genómica y un patrón de aberraciones característico comparado con tumores RE(+).

4. Más allá del estado de RE, el cáncer de mama familiar también se puede dividir en subtipos inmunohistoquímicos que se asemejan a los registrados en tumores esporádicos: subtipo basal, ERBB2, luminal A y luminal B; si bien los tumores *BRCA1* se asocian al subtipo basal y los BRCAX al subtipo luminal A. Cada subtipo presenta un patrón de aberraciones genómicas característico.

5. Los tumores familiares de subtipo basal presentan mayor inestabilidad genómica y un perfil de alteraciones característico, clásicamente asociado a tumores *BRCA1* (+3q, -4p, -4q, -5q, etc.). Además, describimos la amplificación 13q34 como exclusiva de tumores basales y asociada a una sobreexpresión de uno de los genes candidatos (*TFDP1*), promotor de la expresión de genes del ciclo celular (*CCNE*).

6. Los tumores familiares de subtipo luminal B se asocian a un mayor número de amplificaciones de alto nivel. Entre ellas, la amplificación 8p11-p12 fue descrita desde un punto de vista genómico y asociada a una mayor proliferación celular (Ki67) y expresión de genes del ciclo (*CCNE*).

7. Hemos propuesto un modelo integrador para el origen y desarrollo de los diferentes subtipos de cáncer de mama familiar y esporádico basdado en el evento carcinogénico (alteración de *BRCA1*, amplificación/sobreexpresión de ERBB2, amplificación de otras regiones u otros eventos) y del tipo celular en el que tiene lugar (célula troncal o progenitora RE(-) ó RE(+)).

8. Dada la común heterogeneidad en el patrón de aberraciones genómicas entre los subtipos esporádicos y familiares, apoyamos que los distintos subtipos de cáncer de mama familiar pueden tener asociadas diferentes variables clínicas que en un futuro deberían ser objeto de tratamientos y seguimientos más individualizados.

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ANEXO I: MATERIAL SUPLEMENTARIO

A continuación, se detallan aquellas figuras y tablas que han sido citadas a lo largo de la tesis pero que, por su extensión o importancia, se han considerado como material suplementario.

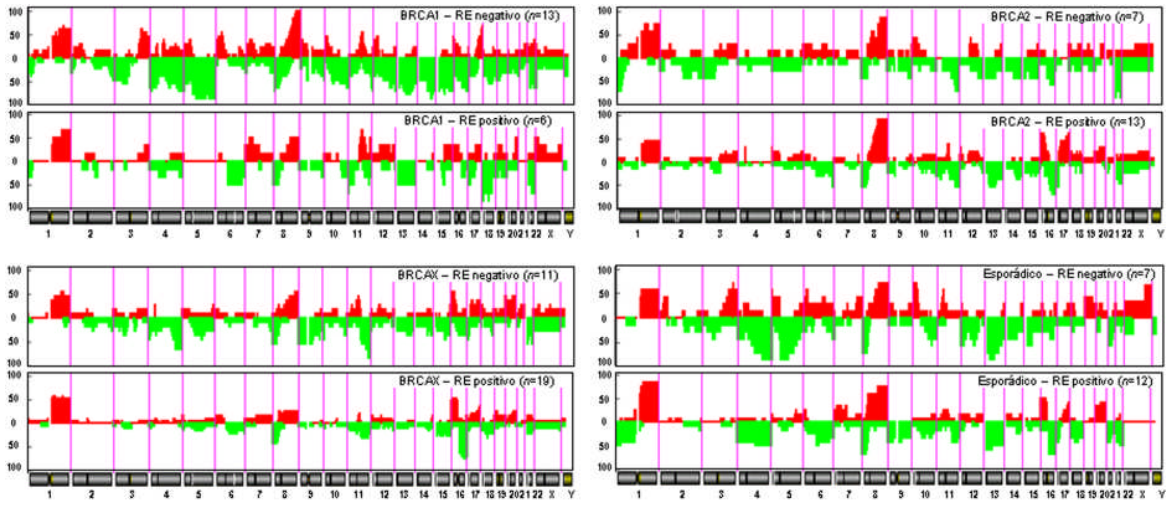

Figura S 1. Patrón de aberraciones genómicas en cada una de las clases tumorales subdivididas por el estado del receptor de estrógenos. La frecuencia de ganancias se muestran en rojo y las de pérdida en verde. Cabe destacar la mayor inestabilidad de los tumores con negatividad para receptor de estrógenos independientemente de la clase tumoral y su tendencia a alterar regiones cromosómicas como 3p, 3q, 4p, 4q y 5q.

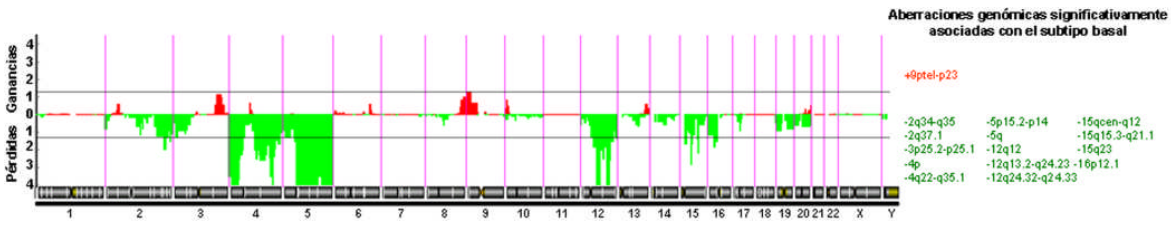

Figura S 2. Aberraciones genómicas significativamente asociadas a los tumores familiares de subtipo basal. Se representa el logaritmo negativo del valor de *P* ajustado de la comparación de los tumores familiares de subtipo basal frente al resto de subtipos. El análisis estadístico se realizó mediante la prueba exacta de Fisher. Las líneas grises señalan los valores de *p* ajustada iguales a 0'05 (-log *p* ajustada =1.3), por lo que aquellas aberraciones cuyos valores superan estas líneas están significativamente asociadas al fenotipo basal, las cuales se detallan en la parte derecha de la figura.

Tabla S 1. Principales datos de los diferentes tumores utilizados en la presente tesis.

Muestra		Mutación en *BRCA1/2*	Edad	Histología	Grado	Estado del RE	Subtipo IHQ	Incluido en los estudios (Sí/No)				
Nº	Código							1	2	3	4	5
Tumores asociados a mutación en *BRCA1*												
1	01T49	E2-185delAG	46	Intraductal	3	-	Basal	S	S	S	N	N
2	01T50	E2-185delAG	48	Intraductal	2	-	Basal	S	S	S	N	N
3	01T142	E18-A1708E	41	/	2	+	No-clasif.	N	N	S	N	N
4	01T152	E2-185delAG	43	Intraductal	3	-	Basal	S	S	S	N	S
5	01T157	E2-185delAG	51	Intraductal	3	+	/	S	S	N	N	N
6	01T162	E19-IVS19+1G/A	29	Intraductal	3	+	/	S	N	N	N	N
7	01T164	E8-157delCT	29	Intraductal	2	-	/	S	N	N	N	N
8	01T167	E2-185delAG	32	Intraductal	3	-	Basal	S	S	S	S	N
9	01T185	E18-A1708E	38	Intraductal	3	-	Basal	S	S	S	N	N
10	02T15	E3-243delA	54	Intraductal	3	+	/	S	N	N	N	N
11	02T19	E11-3600del11	47	Intraductal	2	+	/	S	N	N	N	N
12	02T25	E23-5537delA	43	Intraductal	3	+	No-clasif.	S	S	S	N	N
13	02T124	IVS7-G/A	40	Intraductal	3	-	Basal	S	S	S	N	N
14	02T329 D	IVS6-1G>T	/	Intraductal	/	/	/	S	N	N	N	N
15	02T329 I	IVS6-1G>T	/	Intraductal	/	/	/	S	N	N	N	N
16	02T345	E2-185delAG	34	Intraductal	3	+	/	S	S	N	N	N
17	03T5	E11-3022insTC	39	Lobular	2	-	Luminal B	S	S	S	S	N
18	03T158	E2-185delAG	30	Intraductal	3	-	Basal	S	S	S	N	N
19	03T159	E2-185delAG	31	Intraductal	3	-	Basal	S	S	S	S	S
20	03T236	E18-A1706E	40	Intraductal	3	-	Basal	S	S	S	N	S
21	03T253	E2-185delAG	34	Intraductal	2	+	Luminal B	S	S	S	N	N
22	03T325	IVS-5+1G>4	28	Intraductal	3	-	Basal	S	S	S	N	N
23	03T326	E24-5625G>T	52	Intraductal	3	+	Luminal A	S	S	S	N	N
24	03T327	E11-3598del11	35	Intraductal	3	+	Luminal B	S	S	S	N	N
25	03T329	E2-185delAG	40	Intraductal	3	-	/	S	N	N	N	N
26	03T331	E3-243delA	59	Intraductal	2	-	Luminal B	S	S	S	N	N
27	04T39	IVS18-1G/A	28	Intraductal	3	-	Basal	S	S	S	N	N
Tumores asociados a mutación en *BRCA2*												
28	01T149	E23-9254del5	77	Intraductal	2	+	/	S	N	N	N	N
29	01T150	E14-7336delAA	67	Intraductal	3	+	Luminal B	S	S	S	S	N
30	01T153	E11-3034del4	46	Intraductal	3	-	Basal	S	S	S	S	S
31	01T158	E11-3034del4	34	Intraductal	2	+	Luminal B	S	S	S	N	N
32	01T184	E11-3034del4	41	In situ	In situ	-	/	S	N	N	N	N
33	02T16	E18-8300insTT	45	Lobular	3	+	No-clasif.	S	S	S	N	N
34	02T17	E10-1825delA	39	Intraductal	3	+	No-clasif.	S	S	S	N	N
35	02T18	E11-3374delA	/	In situ	In situ	+	/	S	N	N	N	N
36	02T20	E11-3492insT	44	Intraductal	3	+	No-clasif.	S	S	S	N	N
37	02T23	E11-3036del4	38	Intraductal	2	+	Luminal A	S	S	S	N	N
38	02T26	E11-6857delA	47	Intraductal	2	+	/	S	N	N	N	N
39	02T59	E11-1089insT	36	Intraductal	3	+	No-clasif.	S	S	S	S	N
40	02T60	E11-1089insT	50	/	2	-	Luminal B	S	S	S	N	N
41	02T123	E10-2041delG	30	Intraductal	2	+	Luminal B	S	S	S	N	N
42	03T162	E11-3908delTG	51	Intraductal	3	-	Basal	N	S	S	N	N
43	03T163	E11-K944X	49	Intraductal	3	-	Basal	S	S	S	N	N
44	03T167	E3-E49X	28	Intraductal	2	-	Luminal B	N	S	S	N	N
45	03T168	E11-E1308X	46	Intraductal	1	-	Luminal A	S	S	S	N	N
46	03T238	E18-8260insGA	37	In situ	In situ	+	/	S	S	N	N	N
47	03T322	E3-E49X	55	Intraductal	1	+	Luminal B	S	S	S	N	N
48	TB2627	E11-3917delC	38	Lobular	/	/	/	N	S	N	N	N
49	TB2637	E11-6503delTT	36	Intraductal	3	+	/	N	S	N	N	N
50	TB2765	E11-4392delT	39	Intraductal	3	-	/	N	S	N	N	N
51	TB2767	E10-1133delC	45	Intraductal	/	/	/	N	S	N	N	N
52	TB2769	E16-W2586X	54	Lobular	3	+	/	N	S	N	N	N
53	TB2771	E16-W2586X	45	Intraductal	3	+	/	N	S	N	N	N
54	TB2772	E11-4397delT	39	Intraductal	/	/	/	N	S	N	N	N
55	TB2773	E18-8219delT	39	Lobular	/	/	/	N	S	N	N	N
Tumores no asociados a mutación en *BRCA1/2* ó BRCAX												
56	00T43	-	58	Intraductal	2	+	/	S	N	N	N	N
57	00T44	-	39	Intraductal	1	+	Luminal B	S	S	S	N	N
58	00T46	-	54	In situ	In Situ	-	ERBB2	N	N	S	N	N
59	00T55	-	/	Intraductal	1	+	Luminal A	S	S	S	N	N
60	00T113	-	36	Intraductal	3	-	/	S	N	N	N	N
61	01T6	-	42	Lobular	3	-	ERBB2	S	S	S	N	N
62	01T197	-	43	Intraductal	1	+	Luminal A	S	S	S	N	N
63	01T236	-	39	Intraductal	2	-	Luminal A	S	S	S	N	N
64	01T238	-	41	Intraductal	1	-	ERBB2	S	S	S	N	N

65	01T240	-	/	In situ	In situ	+	Luminal B	S	S	S	N	N
66	01T242	-	54	Intraductal	2	+	Luminal A	S	S	S	N	N
67	01T243	-	49	Intraductal	3	/	/	S	S	N	N	N
68	01T252	-	55	Intraductal	2	+	/	S	S	N	S	S
69	01T264	-	/	Lobular	2	+	Luminal A	S	S	S	S	N
70	01T306	-	44	Intraductal	1	+	Luminal A	S	S	S	N	N
71	02T28	-	/	Intraductal	1	+	/	S	N	N	N	N
72	02T32	-	63	Intraductal	3	+	Luminal B	S	S	S	N	N
73	02T33	-	30	Intraductal	1	+	Luminal A	S	S	S	N	N
74	02T40	-	60	Intraductal	2	+	Luminal B	S	S	S	N	N
75	02T41	-	34	Intraductal	3	-	Luminal B	S	S	S	N	N
76	02T47	-	49	Lobular	2	-	Luminal A	S	S	S	N	N
77	02T255	-	51	Intraductal	3	-	Basal	S	S	S	N	N
78	02T342	-	/	Intraductal	2	-	Basal	S	S	S	N	N
79	02T343	-	/	Intraductal	2	+	/	S	N	N	N	N
80	03T165	-	43	Intraductal	1	+	Luminal A	S	S	S	N	N
81	03T166	-	47	Intraductal	1	+	Luminal A	S	S	S	N	N
82	03T252	-	40	In situ	In situ	+	/	S	N	N	N	N
83	03T270	-	52	Intraductal	1	+	Luminal A	S	S	S	N	N
84	03T282	-	42	Intraductal	1	+	No-clasif.	S	S	S	N	N
85	03T317	-	35	Intraductal	1	+	Luminal B	S	S	S	N	N
86	03T324	-	46	Intraductal	2	+	/	S	S	N	N	N
87	04T34	-	/	Intraductal	1	-	Luminal B	S	S	S	N	N
88	04T35	-	45	Intraductal	1	-	Luminal A	S	S	S	N	N
89	04T41	-	34	Intraductal	3	-	No-clasif.	S	S	S	N	N
90	04T42	-	45	Intraductal	3	+	No-clasif.	S	S	S	S	N
91	04T43	-	39	In situ	In situ	+	Luminal A	S	S	S	N	N
92	04T55	-	46	Intraductal	1	-	ERBB2	S	S	S	N	N
93	04T267	-	/	/	/	/	/	N	N	N	N	S
Tumores esporádicos												
94	TB0001	-	51	Intraductal	3	-	/	N	S	N	N	N
95	TB0004	-	41	Intraductal	3	+	/	N	S	N	N	N
96	TB0010	-	50	Intraductal	3	+	/	N	S	N	N	N
97	TB0017	-	34	Intraductal	3	-	/	N	S	N	N	N
98	TB0022	-	69	Intraductal	2	-	/	N	S	N	N	S
99	TB0026	-	70	Intraductal	2	+	/	N	S	N	N	N
100	TB0031	-	54	Intraductal	3	+	/	N	S	N	N	N
101	TB0032	-	51	Intraductal	3	-	/	N	S	N	N	N
102	TB0033	-	62	Intraductal	3	+	/	N	S	N	N	N
103	TB0038	-	52	Lobular	2	+	/	N	S	N	N	N
104	TB0041	-	50	Intraductal	3	-	/	N	S	N	N	N
105	TB0063	-	51	Intraductal	1	+	/	N	S	N	N	N
106	TB0314	-	60	Intraductal	3	+	/	N	S	N	N	N
107	TB0315	-	41	Intraductal	2	-	/	N	S	N	N	N
108	TB0346	-	47	Intraductal	2	+	/	N	S	N	N	N
109	TB0369	-	36	Intraductal	2	+	/	N	S	N	N	N
110	TB0432	-	62	Intraductal	1	+	/	N	S	N	N	N
111	TB0476	-	74	Intraductal	2	+	/	N	S	N	N	N
112	TB0493	-	52	Intraductal	3	-	/	N	S	N	N	N

El estado del RE se mide en función del porcentaje de células positivas a RE (positivo cuando igual o mayor que 10%).
El subtipo IHQ se ha catalogado en función del panel de marcadores inmunohistoquímicos y el grado histológico utilizados en el estudio número 3.
Los estudios desarrollados han sido: 1, análisis de cCGH de 80 muestras tumorales y su frecuencia y co-ocurrencia de amplificaciones de alto nivel; 2, análisis de aCGH de 93 tumores con una plataforma de 1Mb y en la que se ponen en relación los hallazgos con el estado del RE; 3, análisis de aCGH de 62 tumores poniendo en relación con el subtipo IHQ; 4, análisis del amp8p11-p12 con 9 casos; y 5, análisis del amp13q34 con un total de 7 casos.
El carácter "/" indica falta de datos para esa variable.

ANEXO II: PUBLICACIONES ORIGINADAS POR LA TESIS

En este apartado se adjuntan las publicaciones cuyos resultados se han mostrado a lo largo de la presente tesis:

Clin Cancer Res. 2005 Dec 15;11(24 Pt 1):8577-84.

The accumulation of specific amplifications characterizes two different genomic pathways of evolution of familial breast tumors.

Melchor L, Alvarez S, Honrado E, Palacios J, Barroso A, Díez O, Osorio A, Benítez J.

Int J Cancer. 2007 Feb 1;120(3):714-7.

Genomic analysis of the 8p11-12 amplicon in familial breast cancer.

Melchor L, Garcia MJ, Honrado E, Pole JC, Alvarez S, Edwards PA, Caldas C, Brenton JD, Benítez J.

Clin Cancer Res. 2007 Dec 15;13(24):7305-13.

Estrogen receptor status could modulate the genomic pattern in familial and sporadic breast cancer.

Melchor L, Honrado E, Huang J, Alvarez S, Naylor TL, García MJ, Osorio A, Blesa D, Stratton MR, Weber BL, Cigudosa JC, Rahman N, Nathanson KL, Benítez J.

Oncogene. 2007 Dec 10 [Epub ahead of print]

Distinct genomic aberration patterns are found in familial breast cancer associated with different immunohistochemical subtypes.

Melchor L, Honrado E, García MJ, Alvarez S, Palacios J, Osorio A, Nathanson KL, Benítez J.

Trends in Endocrinology and Metabolism [submitted for publication]

An ingegrative hypothesis about the origin and development of sporadic and familial breast cancer subtypes.

Melchor L, Benítez J.

Human Cancer Biology

The Accumulation of Specific Amplifications Characterizes Two Different Genomic Pathways of Evolution of Familial Breast Tumors

Lorenzo Melchor,[1] Sara Álvarez,[2] Emiliano Honrado,[1] José Palacios,[3] Alicia Barroso,[1] Orland Díez,[4] Ana Osorio,[1] and Javier Benítez[1]

Abstract **Purpose and Methods:** High-level DNA amplifications are recurrently found in breast cancer, and some of them are associated with poor patient prognosis. To determine their frequency and co-occurrence in familial breast cancer, we have analyzed 80 tumors previously characterized for *BRCA1* and *BRCA2* germ-line mutations (26 *BRCA1*, 18 *BRCA2*, and 36 non-*BRCA1/2*) using high-resolution comparative genomic hybridization.

Results: Twenty-one regions were identified as recurrently amplified, such as 8q21-23 (26.25%), 17q22-25 (13.75%), 13q21-31 (12.50%), and 8q24 (11.25%), many of which were altered in each familial breast cancer group. These amplifications defined an amplifier phenotype that is correlated with a higher genomic instability. Based on these amplifications, two different genomic pathways have been established in association with 8q21-23 and/or 17q22-25 and with 13q21-31 amplification. These pathways are associated with specific genomic regions of amplification, carry specific immunohistochemical characteristics coincident with high and low aggressiveness, and have a trend to be associated with *BRCA1* and *BRCA2/X*, respectively.

Conclusion: In summary, our data suggest the existence of two different patterns of evolution, probably common to familial and sporadic breast tumors.

Germ-line mutations of *BRCA1* (OMIM 113705) or *BRCA2* (OMIM 600185) confer an estimated cumulative lifetime risk of 56% to 84% of breast cancer and a 15% to 45% risk of ovarian cancer (1, 2). High-risk families are usually selected based on specific clinical criteria to perform a genomic screening of these genes, but their mutations only explain 30% of familial breast cancers (3). Several candidate loci such as 8p12-p22 (4), 13q21 (5), and 2q32 (6) have been postulated to represent the genetic background of the remaining 70% of families (group named BRCAX) without definitive results.

Immunohistochemical and expression profiles have been defined for familial breast cancers associated with distinct classes of *BRCA* tumors. Briefly, *BRCA1* tumors are high grade, negative for hormone receptors and HER-2, positive for p53, E2F6, cyclins A, B, and E, SKP2, and topoisomerase IIα and display a high expression of P-cadherin (7–10). In contrast, *BRCAX* tumors have a lower grade and an opposite phenotype which is hormone receptor and HER-2 positive, a low proliferation rate, and undetectable P-cadherin expression (9, 10). *BRCA2* tumors show an intermediate phenotype with a higher proliferation rate than *BRCAX*, and positive markers different from *BRCA1* such as cyclins D1 and D3, p27, p16, p21, and cyclin-dependent kinases 4, 2, and 1 (7–10).

Hereditary breast tumors have also been studied characterizing their genomic changes using comparative genomic hybridization (CGH; ref. 11). Some of the genomic changes present in these tumors have been used to build classifiers that distinguish between sporadic and *BRCA1/2* tumors (12, 13) or between *BRCAX* and *BRCA1/BRCA2* tumors (14). This last predictor, which uses high-resolution CGH (HR-CGH), allowed us to identify *BRCAX* cases with a genomic changes profile similar to *BRCA1* tumors, probably due to aberrant methylation of the *BRCA1* promoter (14).

These studies have also shown the existence of common alterations such as high-level DNA amplifications that, in fact, are recurrently found in familial and sporadic breast cancer (15). Some of these amplified regions include known oncogenes, such as *MYC* (8q24), *ERBB2* (17q12), *FLG* (8p12), *CCND1* (11q13), and *IGFR-I/FES* (15q24-q25; refs. 16, 17); some of these oncogenes, such as *MYC* and *ERBB2*, have largely been correlated with poor prognosis (18, 19). However, in other cases, specific high-level amplifications, such as 8q23, 17q23-q25, and 20q11-q13, have been observed at chromosomal sites that do not coincide with the locations of the classic

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Received 6/13/05; revised 9/8/05; accepted 9/30/05.

Grant support: Ministerio de Ciencia y Tecnología grant SAF03-02497 and Red Temática de Investigación Corporativa Centros de Cáncer grant C0-010.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-05-1278

breast cancer oncogenes (20). The comprehensive characterization of some of these amplicons has already started (21, 22) but their meaning remains unclear, especially because some studies have shown that there are tumors with a trend to accumulate high-level DNA amplifications (23–25).

In the present study, we have used HR-CGH to estimate the frequency and distribution of regions with high-level amplifications in familial breast tumors because this genomic event may have important prognostic value. We have classified the cases according to the number of amplifications and defined their genomic characteristics and the immunohistochemical profiles associated with specific amplification pathways.

Materials and Methods

Patients and tumor samples. Eighty breast tumors were collected from patients selected from three centers in Spain: the Spanish National Cancer Centre (CNIO), the Fundación Jimenez Díaz in Madrid, and the Hospital Sant Pau in Barcelona. Patients belonged to families with at least three women affected with breast/ovarian cancer and at least one of them diagnosed before 50 years, or to families with female breast/ovarian cancer and at least one case of male breast cancer. All cases had been studied for mutations in the *BRCA1* and *BRCA2* genes using standard procedures (26). Twenty-six cases presented mutations in the *BRCA1* gene, 18 cases had mutations in the *BRCA2* gene, and 36 cases were negative for germ-line mutations in these genes and were considered as *BRCAX*.

High-resolution comparative genomic hybridization analysis. Genomic DNA was isolated from 4 × 10-μm sections of 80 paraffin-embedded tumors using a commercially available DNase Tissue Kit (Qiagen, Chatsworth, CA) according to the recommendations of the manufacturer. HR-CGH was carried out as described in our recent study that included 72 of these cases, and for genomic studies, we used the 63 regions previously defined (14). Briefly, we chose as the most common minimal regions of involvement 50 regions including imbalances in at least 30% of the *BRCA* cases used to build our previous predictor (14) and with at least three cases defining the cytogenetic thresholds. To include the rest of the genome not fitting the previously defined criteria, we grouped the unselected areas on 13 chromosomal regions. Those chromosomal regions with CGH ratios >1.5 were defined as high-level amplifications and considered as recurrent when they were found in two or more cases.

Tissue microarray and immunohistochemistry. For immunohistochemistry studies, we used a previously published tissue microarray (8, 9) that included 74 of the breast tumors here analyzed by HR-CGH; 23 were *BRCA1*, 18 were *BRCA2*, and 33 were *BRCAX*.

Before tissue microarray construction, total sections of each H&E-stained tumor were evaluated and classified according to the WHO classification. Grade was assessed using the Nottingham grading system.

Immunohistochemical staining was done by the Labeled Streptavidin Biotin method (DAKO, Glostrup, Denmark) with a heat-induced antigen retrieval step. Sections from the tissue array were immersed in boiling 10 mmol/L sodium citrate at pH 6.5 for 2 minutes in a pressure cooker. Antibodies, dilutions, and suppliers are listed in Supplementary Table S1. Two pathologists (E.H. and J.P.) independently evaluated in a blind study the immunohistochemical staining of nine antibodies. The percentage of stained nuclei, independent of the intensity, was scored for estrogen receptor, progesterone receptor, p53, and Ki-67. In the same way, the percentage of cells with cytoplasmic stain was scored for Bcl-2.

We took the mean of the percentage of stained cells as the cutoff point. Thus, when the percentage of stained cells was ≥10%, we considered the tumor as positive for estrogen receptor and progesterone receptor; ≥25%, positive for p53; and ≥70%, positive for Bcl-2. Three categories were defined for Ki-67: 0% to 5%, 6% to 25%, and >25% of stained nuclei.

A tumor was considered to have preserved expression of E-cadherin and catenins (γ-catenin and $p120^{ctn}$) when >75% of the cells showed complete membranous staining of similar intensity as normal breast epithelium (27).

Statistical analysis. We used a nonparametric Mann-Whitney *U* test to identify differences in the number of genomic changes (chromosomal gains or losses) among the patient groups (based on the BRCA class or the number of high-level amplifications). Differences in the frequency of involvement of individual chromosomal regions among the three familial breast cancer classes were tested with Fisher's exact test. The indicated *P* values were calculated using the Stat POMELO (28). This tool is available at http://pomelo.bioinfo.cnio.es. To determine immunohistochemical differences between groups based on the main amplified region, the χ^2 contingency test was used with Fisher's exact test correction when necessary. The SPSS for Windows statistical program (SPSS, Inc., Chicago, IL) was used for this analysis. Hierarchical unsupervised clustering was done using the UPGMA method (28). The statistical test and the clustering are implemented in the GEPAS package (http://bioinfo.cnio.es).

Results

In the present study analyzing 80 tumors by HR-CGH, we have found similar results with our previous work that included 72 of them (14). Briefly, the mean number of changes was higher in *BRCA1* and *BRCA2* than in *BRCAX* tumors. Four chromosomal regions were commonly altered in ~50% of the three *BRCA* groups: gains at 1q and 8q21-23 and losses at 8p21-23 and 11q22-25. There were also specific changes that significantly distinguished each *BRCA* class in the intergroup comparisons ($P < 0.05$, nonadjusted Fisher's exact test). The overall frequencies of changes in the 63 regions are shown in Supplementary Table S2.

Identification of recurrent regions with high-level DNA amplification

We found 21 chromosomal regions recurrently amplified (HR-CGH ratio > 1.5, present in two or more cases). The most frequently involved cytogenetic regions were 8q21-23 (21 of the 80 cases studied), 17q22-25 (11 cases), 13q21-31 (10 cases), 8q24 (9 cases), 12q15-21 (7 cases), and 20q12-13, 15q22-26, and 3q25-26.3 (6 cases each; Fig. 1). The most frequently altered regions were common to all the *BRCA* groups except 17q22-25 and 20q12-13, which were not present in the *BRCA2* mutation carriers.

No amplified regions were found in ~50% of cases of the three groups (39 cases); these cases were named "non-amplifier" (NA) tumors, in contrast to the rest of cases that had at least one amplified region. Based on the median number of amplifications, we established a cutoff that distinguished two further categories: "low-amplifier" (LA) tumors that showed one or two regions with high-level DNA amplifications (lower than the median number; 18 cases) and "high-amplifier" (HA) tumors which had three or more amplified regions (equal to or more than the median number; 23 cases). The distribution of these categories within the distinct *BRCA* classes is shown in Fig. 2. *BRCA1* tumors seem to have a higher trend to accumulate amplifications than *BRCA2* or *BRCAX* tumors although the three groups do not present significant differences. As expected, those tumors with more amplified regions had significantly more genomic alterations. HA tumors have a higher genomic instability than the other phenotypes, and the same occurs with LA tumors

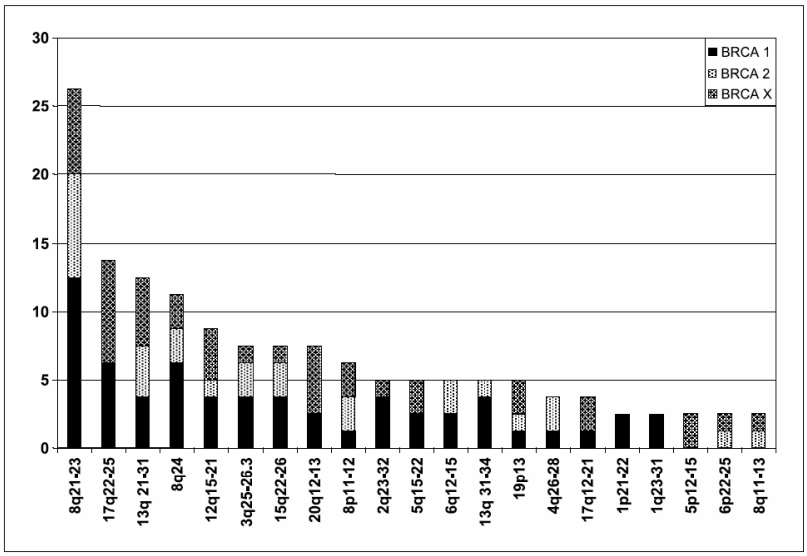

Fig. 1. Distribution of the 21 chromosomal regions recurrently found amplified among the 80 familial breast cancer samples studied.

that present higher instability than NA tumors (Supplementary Fig. S1).

Two different pathways of genomic evolution

Association with specific amplifications. We have analyzed the association of the amplified regions with each other using an unsupervised clustering (Fig. 3A). Two main branches were separated, one of them associated with 13q21-31 amplification which presented a trend to accumulate specific genomic amplifications such as 6q12-15, 5q15-22, 2q23-32, or 4q26-28, and a second one associated with amp8q21-23 and/or 17q22-25, with 15q22-26 or 8q24 regions in the amp8q21-23 group and the 20q12-13 region in the amp17q22-25 tumors. When comparing the frequencies of amplification of the regions between both branches, we observed significant differences (adjusted $P < 0.05$) for 13q21-31 ($P = 0.000$), 8q21-23 ($P = 0.0017$), and 6q12-15 ($P = 0.017$; data not shown). Other genomic changes (gains and losses) were also distributed in a significantly different fashion between both branches (data not shown). When analyzing the distribution of the three BRCA groups, we observed that they were randomly distributed among the two main branches.

Association with immunohistochemical markers. To determine if there were specific characteristics associated with the acquisition of the amplified regions, we defined the immunohistochemical profile by analyzing histologic variables and some markers of proliferation (Ki-67), hormone receptors (estrogen receptor and progesterone receptor), the cell cycle (p53), cell adhesion ($p120^{ctn}$, CAD-E, CAD-P, and G-CAT), and apoptosis (Bcl-2). Statistical comparisons were made to identify the markers that were significantly different in these groups (Table 1). Tumors with amp8q21-23 and/or 17q22-25 were characterized by a higher grade and mitosis number, a high expression of Ki-67, and negative staining for $p120^{ctn}$ and E-cadherin. By contrast, cases with amp13q21-31 mainly presented a low grade and mitosis, a low level of Ki-67, positive staining for hormone receptors (estrogen receptor and progesterone receptor) in nearly 100% of the cases, negative

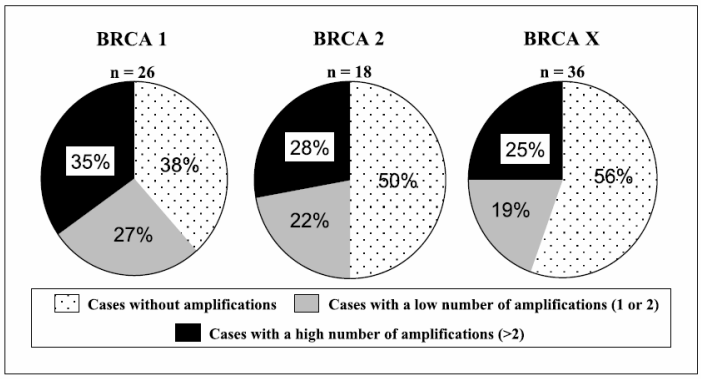

Fig. 2. Distribution of the three genomic phenotypes (HA, LA, and NA tumors) described in this study within the different *BRCA* classes.

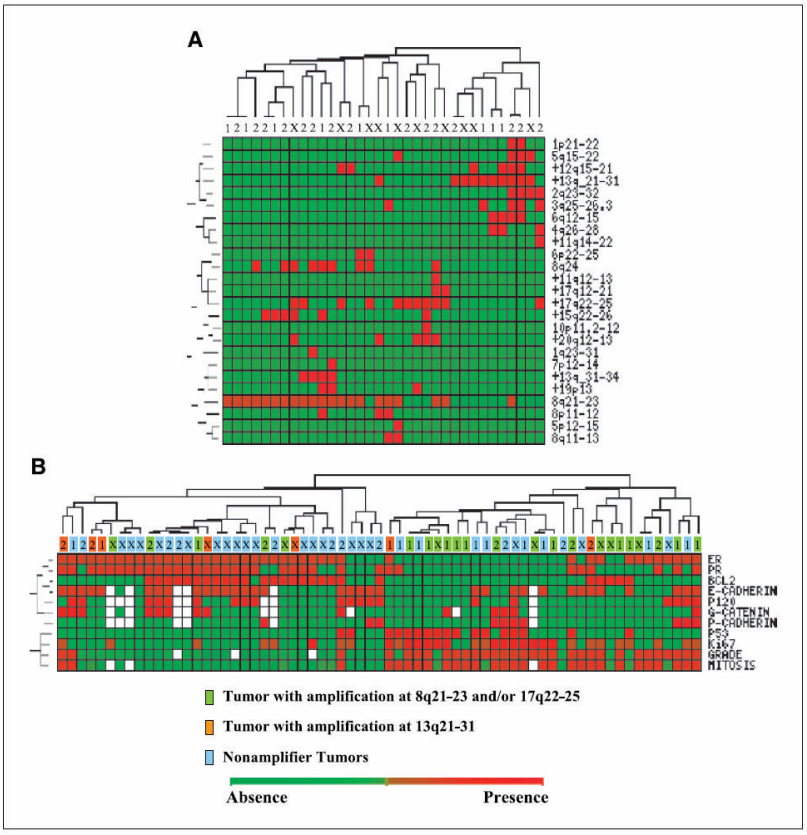

Fig. 3. *A*, unsupervised cluster with the 25 amplified regions (21 recurrent and 4 nonrecurrent) and 34 cases that had at least one amplification at 8q21-23, 13q21-31, or 17q22-25. Red, presence of amplification; green, absence of amplification. Two main groups are represented. The first one with amplification at 13q21-31 and other secondary amplifications (*right*), and the second branch with amplification at 8q21-23, with or without amplification at 17q22-25 and other secondary amplifications (*left*). *B*, unsupervised cluster with immunohistochemical markers in 66 studied cases. Amplified cases without amp8q21-23, 13q21-31, or 17q22-25 were not taken into account. Red, positive expression; green, negative expression; the intensity of the color is a function of the immunohistochemical expression level. White, nonevaluable expression. The right branch presents markers associated with aggressiveness, proliferation, and amplification at 8q21-23; the left branch includes characteristics of low aggressiveness, good prognosis, amplification at 13q21-31, and nonamplifier tumors.

staining for p53 and $p120^{ctn}$, and positive staining for E-cadherin; this latter marker was significantly different from the NA tumors. In addition, NA tumors presented an immunohistochemical phenotype similar to amp13q21-31 tumors although with a lower percentage of cases positive for hormone receptors and negative for $p120^{ctn}$ (Table 1).

Using the studied immunohistochemical variables, we made with all cases an unsupervised cluster to determine if these tumors are well separated (Fig. 3B). The cluster had two main branches: one was associated with high grade and mitosis, a high level of Ki-67, negativity for hormone receptors and Bcl-2, and positive for p53 (right); the other branch showed an opposite phenotype with lower grade and mitosis, a lower level of Ki-67, positivity for hormone receptors and Bcl-2, and negative for p53 (left). The first branch contained the NA *BRCA1* tumors and the majority of cases with amp8q21-23 and/or 17q22-25 (18 cases) independent of the *BRCA* type. In the left branch, there was a mix of NA *BRCA2/X* tumors (23 cases), amp13q21-31 (5 cases), and amp8q21-23 (5 cases).

Discussion

In the present study, we have established the most frequently found somatic alterations in familial breast tumors by analyzing 63 chromosomal regions by HR-CGH. We have defined the existence of a genomic phenotype that is characterized by the accumulation of DNA amplifications that are commonly found in the three *BRCA* groups. Finally, we have shown three major amplified regions that define two different genomic pathways that seem to be associated with specific immunohistochemical characteristics and prognosis.

We have found four common genomic alterations present in the three classes of *BRCA* tumors: gains at 1q and 8q21-23 and losses at 8p21-23 and 11q22-25. These alterations coincide with those described in other studies on *BRCA* mutation carriers (11, 13) and with our previous study, although the gain at 8q21-23 had lower frequency in *BRCAX* cases (14). It is interesting to note that van Beers et al. (13) included 80 regions with alterations defined by CGH in a similar way as described in the present study, and the majority of these regions coincide with ours. Therefore, these altered regions could represent a core of abnormalities common to familial and sporadic breast cancer.

We also found specific regions associated with the three *BRCA* groups (Supplementary Table S2). However, only loss at 5q11-23 in *BRCA1* and gain at 3q11-23 in *BRCA2* coincide with the specific changes associated with some of the *BRCA* classes defined by van Beers et al. (13). These specific chromosomal regions warrant further analysis.

Finally, we have defined three different genomic phenotypes: NA, with no amplification (48% of the total number of

samples); LA, with one or two amplified regions (23%); and HA, with three or more amplifications (29%). As expected, tumors with high-level DNA amplifications presented higher instability than NA tumors. These results support the idea that tumors with a high genomic instability have an increased probability to develop multiple amplifications (18). In this scheme, LA tumors would represent the first level of genomic instability to develop more amplifications.

Different pathways of genomic evolution. Among the 21 regions with high-level DNA amplification that we have identified on familial breast cancer, three amplified regions characterize two different genomic pathways. 8q21-23 and 13q21-31 are the two regions with the highest frequency of amplifications in both LA and HA tumors and may represent the earliest amplified regions that drive the tumors through distinct pathways of evolution (Fig. 4). In contrast, amp17q22-25, the second most frequent amplification, may be associated with tumor progression because it seemed to be significantly more frequent in HA tumors and was often amplified together with 8q21-23 (Fig. 4). By using unsupervised clustering, we

Table 1. Comparison of immunohistochemical variables among familial breast tumors with amplification at 8q21-23 and/or 17q22-25, amplification at 13q21-31, and without amplifications

	Cases with amp8q21-23 and/or amp17q22-25	P^*	Cases with amp13q21-31	$P^\dagger$	Nonamplifier cases	$P^\ddagger$
	n (%)		*n* (%)		*n* (%)	
Grade						
1	2 (9.1)	NS	1 (11.1)	NS	13 (37.1)	0.028
2	6 (27.3)		4 (44.4)		9 (25.7)	
3	14 (63.6)		4 (44.4)		13 (37.1)	
Mitosis						
1	3 (15.0)	NS	4 (44.4)	NS	20 (55.6)	0.006
2	4 (20.0)		1 (11.1)		4 (11.1)	
3	13 (65.0)		4 (44.4)		12 (33.3)	
Ki-67						
0-5%	7 (30.4)	NS	5 (62.5)	NS	24 (64.9)	0.040
6-25%	9 (39.1)		2 (25.0)		7 (18.9)	
>25%	7 (30.4)		1 (12.5)		6 (16.2)	
Estrogen receptor						
Negative	11 (47.8)	0.028	0	NS (0.085)	14 (36.8)	NS
Positive	12 (52.2)		8 (100)		24 (63.2)	
Progesterone receptor						
Negative	14 (60.9)	0.037	1 (12.5)	NS (0.115)	18 (47.4)	NS
Positive	9 (39.1)		7 (87.5)		20 (52.6)	
p53						
Negative	16 (69.6)	NS	7 (87.5)	NS	28 (75.7)	NS
Positive	7 (30.4)		1 (12.5)		9 (24.3)	
$p120^{ctn}$						
Negative	15 (78.9)	NS	8 (100)	0.013	16 (50.0)	0.023
Positive	4 (21.1)		0		16 (50.0)	
E-Cadherin						
Negative	12 (60.0)	NS	3 (37.5)	NS	12 (36.4)	0.047
Positive	8 (40.0)		5 (62.5)		21 (63.6)	
P-Cadherin						
Negative	16 (80.0)	NS	8 (100)	NS	26 (81.3)	NS
Positive	4 (20.0)		0		6 (18.8)	
γ-Catenin						
Negative	13 (72.2)	NS	6 (75.0)	NS	25 (78.1)	NS
Positive	5 (27.8)		2 (25.0)		7 (21.9)	
Bcl-2						
Negative	14 (63.6)	NS	5 (62.5)	NS	22 (59.5)	NS
Positive	8 (36.4)		3 (37.5)		15 (40.5)	

NOTE: All *P* values were obtained by the χ^2 contingency test using Fisher's exact test correction when necessary. $P < 0.05$ was considered significant. NS, non-significant differences.

*P obtained by comparison of cases with amp8q/17q versus amp13q.

†P obtained by comparison of cases with amp13q versus nonamplifier tumors.

‡P obtained by comparison of cases with amp8q/17q versus nonamplifier tumors.

could confirm these points: we observed that the branch represented by amp8q21-23 is associated with specific regions such as 15q22-26 and 8q24, and that the second branch defined by amp13q21-31 is associated with 6q12-15, 5q15-22, 2q23-32, and 4q26-28 (Fig. 3A). Therefore, the pathways defined by 13q21-31 and 8q21-23, with or without 17q22-25, might influence the future accumulation of other specific amplified regions and would determine the genomic evolution of these tumors. Genomic evolution is a frequent event in breast cancer (25, 29–32). In this way, Courjal and Theillet (29) and Courjal et al. (32) described the genetic evolution of a set of sporadic breast tumors based on amplified regions. Because the amplified regions they found affected the same chromosomes and in similar regions as in our genomic pathways, we think that 13q21-31, 8q21-23, and 17q22-25 may be common genomic amplification pathways in breast cancer.

Because of the genomic differences between the two pathways, we have tried to define the immunohistochemical characteristics associated with each one of them. We analyzed 11 immunohistochemical variables and found that tumors with amp13q21-31 presented a profile defined by positive estrogen receptor and progesterone receptor staining in 100% and 90% of cases, respectively, a low Ki-67 expression, and negative p53 and $p120^{ctn}$ staining (Table 1). The majority of these markers have been previously correlated with good prognosis and low malignant potential in *BRCA2* and *BRCAX* familial tumors (7–10), and although the number of tumors here analyzed is small, the majority correspond to these subtypes. In contrast, the group associated with amp8q21-23 with or without amp17q22-25 showed immunohistochemical variables of aggressiveness, such as a high grade and mitosis number, high expression of Ki-67, and negative expression of hormone receptors and E-cadherin. Moreover, recent studies by our group and others have shown that the immunohistochemical characteristics of *BRCA1* tumors are in general represented by this profile (7–10), and although in the amp8q21-31/17q22-25 group all three subtypes are represented, the majority are *BRCA1* tumors. Regarding NA tumors, the immunohistochemical profile was similar to that of the amp13q21-31 group.

The unsupervised cluster showed the same trend (Fig. 3B): the right branch included phenotypic markers of aggressiveness and mainly contained NA *BRCA1* tumors (8 cases) and *BRCA1* and *BRCA2/X* tumors with amp8q21-31/17q22-25 (10 and 8 cases, respectively); in the left branch, we found NA *BRCA2/X* tumors (23 cases) and *BRCA1* and *BRCA2/X* tumors with amp13q21-31 (1 and 4 cases, respectively). Only a minority of cases was incorrectly located according to the amplified region or *BRCA* type. All these data suggest that the genetic mutation in familial breast tumors is mainly responsible for the immunohistochemical phenotype, although a small group can present different phenotypes (more or less aggressive) probably due to their own genetic background. During tumor evolution, the genetic phenotype may induce a set of genomic changes through two main pathways: amp13q21-31, which is associated with less aggressive tumors and good prognosis, and amplification of the 8q21-23/17q22-25 region, which is associated with highly aggressive tumors and bad prognosis. Thus, *BRCAX* cases that amplify 8q21-23 and/or 17q22-25 may represent a more aggressive subgroup within the heterogeneous population of *BRCAX* tumors, and this should be taken into account in future searches for genes responsible for BRCAX.

Why this occurs is currently unknown but different studies have pointed out that the gain of 8q is a recurrent event in sporadic breast cancer with poor prognosis (23, 24, 33, 34). Our results support this correlation and suggest that 8q amplification may be a universal bad prognostic marker for breast cancer.

The same occurs with the amplification 17q22-25, which is distal to *ERBB2* and *BRCA1* genes and seems to be a major amplification site in sporadic breast cancer (20, 29, 35–37). This amplified region has also been correlated with poor patient prognosis (24, 33) and detailed characterizations of

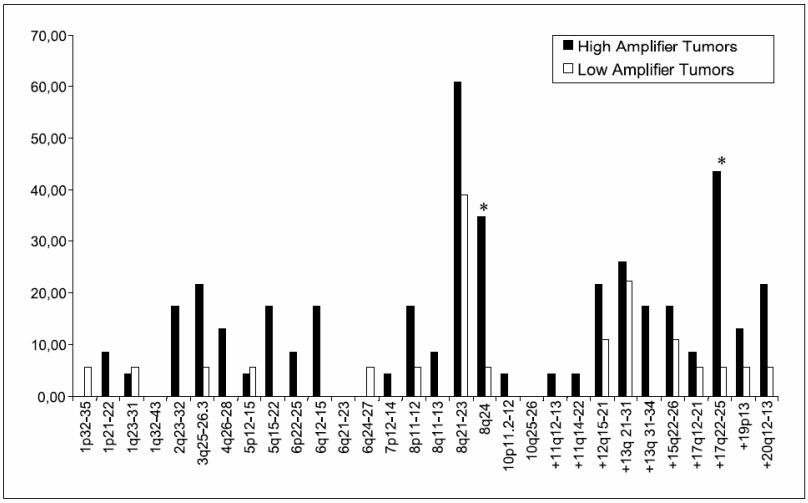

Fig. 4. Frequency of the 21 recurrent amplified regions found in the HA (*black columns*) and LA tumors (*white columns*). Amplifications at 8q21-23 and 13q21-31 are present with a high frequency in both groups. However, amplifications at 8q24 and 17q22-25 occur more frequently on HA tumors and may be considered as late alterations in the tumor development. *, significant differences in the statistical comparison of the frequencies between HA and LA tumors.

the amplification and overexpression of genes located at this region have already been reported (21, 38–41).

Finally, the amp13q21-31 group and its correlation with good prognosis still have to be studied in detail. Different candidate genes are located in this region, such as protocadherins (*PCDH9* and *PCDH17*), mitotic control protein genes (*KIAA1008*), Kruppel-like transcription factors (*KLF5* and *KLF12*), transcription regulatory function genes (*LMO7*), or inhibitors of natural killer activity and prostaglandin synthesis during pregnancy (*PIBF1*; refs. 5, 42, 43). 13q21-31 amplification has also been reported in breast cancer cell lines (20, 44, 45) but little is yet known on its role. Our findings about the recurrence of this amplification in all *BRCA* groups, but mainly in *BRCA2* and *BRCAX*, the strong correlation with estrogen receptor–positive tumors (100%), and the good prognosis immunohistochemical profile indicate that this amplified region could serve as a marker for tumor evolution and follow-up of the patient.

In summary, we have defined 21 recurrent amplification sites and described an amplifier phenotype that is probably common not only to familial but also to sporadic breast tumors and that is correlated with a higher genomic instability. In addition, two different genomic pathways associated with 8q/17q and 13q amplification have been established. They are associated with specific genomic regions of amplification, carry specific immunohistochemical characteristics, and have a trend to be associated with *BRCA1* and *BRCA2/X*, respectively. According to these data, tumors with 13q amplifications are associated with low aggressiveness and good prognosis whereas 8q/17q amplification defines tumors with high aggressiveness and poor prognosis. Because these amplifications have been previously identified in sporadic breast tumors and correlated with prognosis, the two pathways may represent a general mechanism of evolution in breast tumors and may contain key genes for tumor evolution.

Acknowledgments

We thank the Spanish National Tumor Bank Network, the Immunohistological Unit of the Spanish National Cancer Centre, and Carmen Martin from the Cytogenetics Unit for their technical support; Roger Milne and Ramón Díaz-Uriarte for their assistance in the statistical analysis; and Miguel Urioste, Laura Valle, and Raquel Rodríguez for the selection of families.

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Int. J. Cancer: **120,** 714–717 (2006)

SHORT REPORT

Genomic analysis of the 8p11-12 amplicon in familial breast cancer

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Amplification of 8p11-12 has been recurrently reported in sporadic breast cancer. These studies define a complex molecular structure with a set of minimal amplified regions, and different putative oncogenes that show a strong correlation between amplification and over-expression such as *ZNF703/FLJ14299, SPFH2/C8orf2, BRF2* and *RAB11FIP*. However, none of these studies were carried out on familial breast malignancies. We have studied the incidence, molecular features and clinical value of this amplification in familial breast tumors associated with *BRCA1*, *BRCA2* and non-*BRCA1/2* gene mutations. We detected 9 out of 80 familial tumors with this amplicon by chromosomal comparative genomic hybridization. Next, we used a high-resolution comparative genomic hybridization array covering the 8p11-12 region to characterize this chromosomal region. This approach allowed us to define 2 cores of common amplification that largely overlap with those reported in sporadic tumors. Our findings confirm the molecular complexity of this chromosomal region and indicate that this genomic event is a common alteration in breast cancer, present not only in sporadic but also in familial tumors. Finally, we found correlation between the 8p11-12 amplification and proliferation (Ki-67) and cyclin E expression, which further proves in familial tumors the poor prognosis association previously reported in sporadic breast cancer.

Key words: 8p11-12 amplification; array CGH; familial breast cancer

The short arm of chromosome 8 is frequently altered in solid and hematological human tumors.[1–4] Amplification at 8p11-12 has been reported in 10–15% of sporadic breast cancer,[3,5,6] although according to recent studies, using high-resolution array comparative genomic hybridization (aCGH) techniques, this frequency may be as high as 25%.[7,8]

Recently, 3 different groups have characterized the 8p11-12 amplification in sporadic breast tumors and breast cancer cell lines using aCGH, to define the region and describe relevant genes that map in this chromosomal location.[7–9] Gelsi-Boyer *et al.*[9] defined 4 minimal amplicons in the 8p11-12 amplified region (Fig. 1). The most telomeric core of amplification (A1) spans 1.27 Mb and comprises the entire minimal region previously described by Garcia *et al.*[8]; this minimal region of common amplification is 1 Mb long and contains several candidate oncogenes (Fig. 1). This narrow region was similarly reported by a third group that suggests breakpoints and complex chromosomal rearrangements within the *NRG1* locus (31.38–32.70 Mb) as likely mechanisms involved in the amplification.[7] The second amplicon (A2), centromeric to A1, has a length of around 800 Kb and contains among other genes *FGFR1*. This gene has been previously proposed as candidate oncogene but its role as target of the 8p12 amplification remains to be established.[3–5,11–16] Two more amplicons, A3 and A4, centromeric to the previous ones and with lengths of 1.25 Mb and 460 Kb, respectively, were also described. Candidate oncogenes at each of these amplicons are listed in Figure 1.

Two regions of recurrent breakpoints in 8p have also been described in breast cancer: a telomeric cluster of breakpoints (BPC1) associated with rearrangements at the *NRG1* locus (31.38–32.70 Mb),[9,10,17] and a centromeric cluster of breakpoints (BPC2) proximal to the *NRG1* and *UNC5D* genes[8–10] (Fig. 1). The telomeric region of 8p distal to these breakpoints is frequently deleted as a result of these breaks.[8–10] This pattern of molecular complexity has been recently reported by us, not only in breast cancer cell lines but also in colon and pancreatic cancer cell lines.[10]

All these data suggest that the 8p11-12 region is prone to present breaks and complex rearrangements, and could therefore participate in oncogenesis *via* inactivation of one or several potential tumor suppressor genes, and/or *via* amplification and overexpression of candidate oncogenes.

We have previously reported 9 cases (11.25%) showing 8p11-12 amplification in a set of 80 familial breast tumors (including 26 *BRCA1*, 18 *BRCA2* and 36 non-*BRCA1/2* tumors), using chromosomal comparative genomic hybridization (cCGH).[18] Because familial tumors present different genetic and immunohistochemical (IHC) profiles among themselves and also with respect to sporadic tumors, we decided to investigate the features of this amplicon at the molecular level, to establish whether it is a common event in all breast cancer types.

Patients included in our previous study[18] belonged to families with at least 3 women affected with breast/ovarian cancer and at least 1 of them diagnosed before 50 years, or to families with female breast/ovarian cancer and at least with 1 case of male breast cancer. All cases were studied for mutations in *BRCA1* and *BRCA2* genes and for large rearrangements alterations using standard procedures.[19] Those that were negative for this mutational screening were considered as non-*BRCA1/2* patients. We extracted DNA from paraffin-embedded tissue from the 9 familial

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0020-7136/suppmat.

JDB is a Cancer Research UK Senior Clinical Research Fellow, Cancer Research, UK.

Grant sponsor: Spanish Ministries of Health and Science; Grant numbers: FIS04-2224, SAF03-02497; LM Grant sponsor: Spanish Ministry of Science; Grant number: AP-2004-0448; EH Grant sponsor: Spanish Association Against Cancer (AECC); Grant sponsor: Cancer Research, UK.

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Received 17 July 2006; Accepted 30 August 2006
DOI 10.1002/ijc.22354
Published online 9 November 2006 in Wiley InterScience (www.interscience.wiley.com).

Publication of the International Union Against Cancer

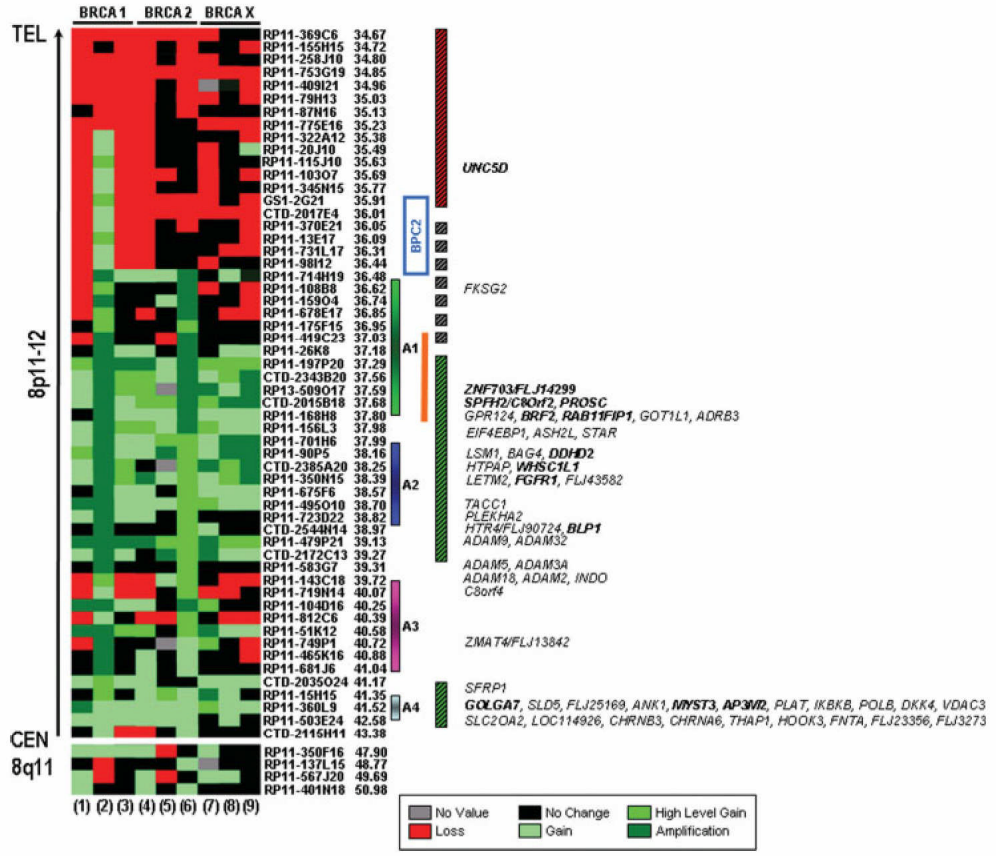

Figure 1 – Array-CGH profile across the 8p11-12 region. The 9 samples are represented in columns: 3 BRCA1 (1–3), 3 BRCA2 (4–6) and 3 BRCAX (7–9). Each row represents a clone on the array. Clones have been ordered by genome position according to the NCBI Build 35 from the most distal ones (top) to the most centromeric ones (bottom). The midpoint position (Mb) of each clone is indicated beside the clone name, as well as the genes mapping in the region. Colors represent discrete values according to the aCGH ratio: red indicates loss (ratios <0.8), black implies no change (0.8–1.2) and a green scale represents distinct levels of gains. Light green represents simple gain (1.2–1.5), midtone green is high-level gain (1.5–1.75) and dark green represents BAC amplification (>1.75). Grey cells correspond to data rejected after quality tests for signal intensity and replicate reproducibility. Genes in bold font present a good correlation between amplification and expression levels according to previous studies. Left colored thick bars represent the 4 cores of amplification described by Gelsi-Boyer *et al.* (A1, A2, A3, A4).[9] The orange bar defines the 1-Mb minimal amplicon reported by us in a previous study.[8] The blue box represents the cluster of breakpoints proximal to the *UNC5D* gene reported by Gelsi-Boyer *et al.* (BPC2).[9,10] Finally, the further right colored striped bars represent the region of loss distal to breakpoints (red box), the area where breaks mainly occurred (discontinuous grey boxes) and the 2 regions of gain/amplification (green boxes) we describe.

breast tumor samples in our collection (3 BRCA1, 3 BRCA2 and 3 non-BRCA1/2) that displayed amplification at the 8p11-12 region. We used a xylene treatment and sodium thiocyanate incubation before proteinase K digestion and phenol chloroform extraction. We then hybridized these cases onto a new version of a previously used in-house BAC-array.[8,10,17] Briefly, this platform comprised BACs ~10 Mb apart across the whole genome; 1.5 Mb apart over chromosome 8; and at near-tiling-path density over 8p11-12. Interestingly, its resolution at 8p11-12 was further improved by adding 30 more clones to fill in existing gaps at the region. A total number of 91 BACs (*versus* 61 in the previous set) spans over 9.5 Mb at 8p11-12 between positions 31.03 Mb (RP11-473A17) and 43.38 Mb (CTD-2115H11). Name, position, size and accession number for these clones are available in Supplementary Table I. DNA labeling and hybridization as well as image acquisition and data analysis were done as previously published.[8] Clinical and IHC data of the 80 cases studied by cCGH were collected from earlier studies by our group.[20,21]

The genomic characterization of the 8p11-12 region in the 9 cases with amplification by means of hybridization on the high-resolution aCGH platform is summarized in Figure 1. The majority of the cases did not show any rearrangements at the previously described cluster of breakpoints BPC1, which has been associated with the *NRG1* locus. In contrast, most of breakages occurred roughly between BACs CTD-2017E4 (36.01 Mb) and RP11-26K8 (37.18 Mb). Genomic losses telomeric to the breakpoints and gain/amplification centromeric to the breakpoints were observed, reproducing the genomic imbalances already defined at this chromosomal region.[8,9] This region of breakpoints overlaps with the previously described cluster of breakpoints centromeric to the *UNC5D* gene, BPC2 (Fig. 1).[9,10] These findings highlight this area as prone to develop breaks in different types of tumors, including

TABLE I – CORRELATIONS BETWEEN 8p11-12 AMPLIFICATION AND IMMUNOHISTOCHEMICAL AND CLINICAL FEATURES

	No amplification, *n* (%)	8p11-12 amplification,[1] *n* (%)	*p**
Age (years)			
<44	25 (45.5)	5 (55.6)	NS
≥44	30 (54.5)	4 (44.4)	
SBR grade			
1	17 (28.3)	1 (11.1)	NS
2	17 (28.3)	2 (22.2)	
3	26 (43.3)	6 (66.7)	
Estrogen receptor			
<10	24 (35.8)	4 (44.4)	NS
≥10	43 (64.2)	5 (55.6)	
Progesterone receptor			
<10	32 (47.8)	5 (55.6)	NS
≥10	35 (52.2)	4 (44.4)	
p53			
<25	51 (77.3)	7 (77.8)	NS
≥25	15 (22.7)	2 (22.2)	
Ki-67			
<20	49 (74.2)	3 (33.3)	0.013
≥20	17 (25.8)	6 (66.7)	
Cyclin D1			
<25	32 (51.6)	4 (50.0)	NS
≥25	30 (48.4)	4 (50.0)	
Cyclin D3			
<10	34 (56.7)	5 (71.4)	NS
≥10	26 (43.3)	2 (28.6)	
Cyclin E			
<10	46 (74.2)	3 (37.5)	0.033
≥10	16 (25.8)	5 (62.5)	
Cyclin A			
<10	23 (37.7)	1 (12.5)	NS
≥10	38 (62.3)	7 (87.5)	
Cyclin B1			
<10	41 (68.3)	3 (42.9)	NS
≥10	19 (31.7)	4 (57.1)	
p16			
<50	22 (40.0)	3 (37.5)	NS
≥50	33 (60.0)	5 (62.5)	
p21			
<10	35 (57.4)	2 (25.0)	NS
≥10	26 (42.6)	6 (75.0)	
p27			
<50	26 (42.6)	4 (50.0)	NS
≥50	35 (57.4)	4 (50.0)	

[1]Cases whose 8p11-12 amplification was defined by cCGH and studied in the present work.

*p-value defined by Pearson's χ^2 test. NS, non significant.

familial breast cancer. Regarding amplified regions, all cases showed distinct levels of gains that were grouped in 2 main subregions (Fig. 1). The first one has a length of 2.13 Mb, and comprises the proximal half of A1, the whole of A2, and a small region of around 100 Kb between A2 and A3. This genomic area contains genes such as *ZNF703/FLJ14299, SPFH2/C8orf2*, *PROSC, DDHD2*, *WHSC1L1* and *FGFR1*, all of them previously reported as genes of interest for further functional analysis because of their amplification-over-expression correlation.[7–9] It is important to note that this chromosomal region includes the minimal 1 Mb amplicon already defined by us,[8] supporting the candidate role of the genes located there. The second subregion encompasses the A4 core of amplification, has a length of 1.41 Mb, shows a lower level of gain and contains other genes already described as relevant such as *GOLGA7*, *MYST3* and *AP3M2*.[1,9] Interestingly, the majority of cases in our sample set did not present amplification at the A3 region. This region was amplified in only 2 cases (cases 2 and 6) that presented large and continuous amplifications from about positions 35 and 36 Mb up to the centromere (Fig. 1). No differences in the pattern and distribution of the amplifications were observed among the 3 types of familial breast tumors (BRCA1, BRCA2 and non-BRCA1/2).

Amplification of 8p11-12 has been associated with high proliferation (high histological grade and Ki-67 expression)[9] and an adverse effect on survival in breast cancer.[7,9] To evaluate these clinical associations in our series, we compared different IHC markers and clinical variables between the 9 tumors with this amplification and those cases without this genomic event (68 samples) (Table I). We found that tumors with 8p11-12 amplification had significantly higher Ki-67 and cyclin E expression ($p = 0.013$ and 0.033, respectively). Moreover, we observed a trend to present high grade, high expression of other cell cycle markers (such as cyclin A or B1) and an early age of onset in tumors with the amplification. These differences were not statistically significant, probably due to the low number of cases (Table I). These clinical and IHC features have been largely associated with tumor progression, proliferation and poor patient prognosis.[22–27] Therefore our data indicate that this amplified region would present clinical prognostic value in familial breast cancer, as it has been shown for sporadic breast tumors. Interestingly, the region A2 described as the amplification core associated with the most aggressive tumor behavior[9] was amplified in all of our cases.

In summary, to our knowledge, this is the first time that the 8p11-12 amplification has been reported and analyzed in detail in familial breast cancer. We have defined 2 common regions of amplification that greatly overlap with the minimal regions of amplifications previously described in sporadic tumors. We also found a cluster of breakpoints centromeric to the *UNC5D* gene, similarly to what has been reported for sporadic neoplasms. Therefore our findings in a selected group of familial tumors confirm the molecular complexity of the 8p11-12 chromosomal region and suggest that these alterations, and probably some gene(s) mapping in these regions, are common in breast cancer pathogenesis, independent of the tumor type. Finally, we have found that the presence of this amplification is associated with high proliferation (Ki67) and cyclin E expression, which further supports the clinical value of this aberrations found in sporadic breast cancer.

Acknowledgements

We thank the Spanish National Tumor Bank Network, the Familial Cancer Unit, the Immunohistological Unit and the Molecular Cytogenetics Group of the Spanish National Cancer Center for their technical support.

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Human Cancer Biology

Estrogen Receptor Status Could Modulate the Genomic Pattern in Familial and Sporadic Breast Cancer

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Abstract

Purpose: Familial breast cancer represents 5% to 10% of all breast tumors. Mutations in the two known major breast cancer susceptibility genes, *BRCA1* and *BRCA2*, account for a minority of familial breast cancer, whereas families without mutations in these genes (BRCAX group) account for 70% of familial breast cancer cases.

Experimental Design: To better characterize and define the genomic differences between the three classes of familial tumors and sporadic malignancies, we have analyzed 19 BRCA1, 24 BRCA2, and 31 BRCAX samples from familial breast cancer patients and 19 sporadic breast tumors using a 1-Mb resolution bacterial artificial chromosome array-based comparative genomic hybridization.

Results: We found that BRCA1/2 tumors showed a higher genomic instability than BRCAX and sporadic cancers. There were common genomic alterations present in all breast cancer groups, such as gains of 1q and 16p or losses of 8ptel-p12 and 16q. We found that the presence/absence of the estrogen receptor (ER) may play a crucial role in driving tumor development through distinct genomic pathways independently of the tumor type (sporadic or familial) and mutation status (BRCA1 or BRCA2). ER^- tumors presented higher genomic instability and different altered regions than ER^+ ones.

Conclusions: According to our results, the BRCA gene mutation status (mainly BRCA1) would contribute to the genomic profile of abnormalities by increasing or modulating the genome instability.

Two major genes associated with susceptibility to hereditary breast cancer have been identified to date: *BRCA1* and *BRCA2* (1, 2). Inheritance of a mutation in these genes confers an increased lifetime risk of breast cancer (60-85%) and ovarian cancers (15-40%; ref. 3), although these genes only explain ~25% of breast cancers within high-risk families (4, 5). Some groups have tried to find putative BRCAX gene(s) using linkage analysis to explain the genetic background of some of the remaining high-risk families but without conclusive results (6–9).

A large amount of data have been presented showing that breast tumors from patients with germ-line mutations in the *BRCA1* and *BRCA2* genes present morphologic and genetic differences and also differ from BRCAX tumors and sporadic breast cancer cases (see refs. 10, 11 for review). These data suggest that hereditary mutations in *BRCA1* and *BRCA2* lead to breast cancer development through different signaling pathways. One feature of solid tumors, and thus of breast cancer, is genomic instability, which involves chromosomal changes, such as DNA gains or losses. To understand these changes, many groups have pursued tumor genome profiling of different classes of breast cancer. By chromosomal comparative genomic hybridization (cCGH; ref. 12), *BRCA1*-associated tumors are characterized by a high frequency of losses of 5q, 4q, 4p, 2q, and 12q; whereas *BRCA2*-associated tumors present frequent losses of 13q and 6q and gains of 17q22-q24 (13). In addition, and because of the recent development of classifiers based on specific DNA copy number alterations of each tumor class (14–16), cCGH profiling has been proposed as a potential diagnostic tool.

More recently, array-based CGH (aCGH) has become widely used, providing higher resolution and flexibility than cCGH (see refs. 17–19 for review). The single analysis of familial breast cancer using aCGH published to date has confirmed

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Received 3/28/07; revised 7/5/07; accepted 9/14/07.

Grant support: Spanish Ministry of Education and Science grant FPU AP-2004-0448 (L. Melchor), ICRETT/05/063 and European Molecular Biology Organization Short-Term Fellowship ASTF 162-05, Spanish Ministry of Education and Science projects SAF-03-02497 and SAF-06-06149, and Breast Cancer Research Foundation (K.L. Nathanson).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-07-0711

some previous findings, such as the higher genomic instability and specific losses of 4p, 4q, and 5q observed in *BRCA1*-associated tumors and frequent gains of 17q24 found in breast cancers associated with *BRCA2* mutations (20). The authors also described a set of chromosomal regions that correctly discriminated among BRCA1, BRCA2, and sporadic breast tumors. However, BRCAX cases were not included in this study, and the results need to be confirmed in larger and independent series.

In the present study, we aimed to, first, establish the genomic profile of sporadic and BRCA1, BRCA2, and BRCAX familial breast cancers using a 1-Mb resolution bacterial artificial chromosome (BAC) array platform and, second, identify regions commonly altered in all groups and regions that are class specific. Finally, because the array platform we used was the same as the one implemented by Jönsson et al., we tried to validate the aberrant regions they proposed in our tumor sample set.

Materials and Methods

Patients and tumor samples. We collected formalin-fixed, paraffin-embedded (FFPE) breast tumor tissues from 74 patients. These patients were selected from families with at least three women affected with breast/ovarian cancer and at least one of them diagnosed before 50 years of age or from families with female breast/ovarian cancer and at least one case of male breast cancer. All cases were studied for mutations and large rearrangements in the BRCA genes using standard procedures (4, 21). Nineteen cases had mutations in the *BRCA1* gene, 24 patients presented mutations in the *BRCA2* gene, and 31 cases were negative for germ-line mutations in the BRCA genes and therefore denoted as BRCAX tumors. Eight of the breast cancers with *BRCA2* mutations were provided by the Breast Cancer Genetics Team, Institute of Cancer Research (Sutton, Surrey, United Kingdom). We also included a set of snap-frozen sporadic breast tumors from 19 unselected patients without a family history of breast cancer. All data on sporadic samples were provided by the University of Pennsylvania and included in a previous study (22). BRCA gene mutation status, age at diagnosis, type of carcinoma, histologic grade, and estrogen receptor (ER) status are provided in Supplementary Table S1. ER status was considered as positive when the percentage of stained cells was ≥10% in an immunohistochemistry analysis, as previously reported (23, 24).

DNA extraction. Genomic DNA from frozen tissue sections was extracted using proteinase K digestion followed by phenol/chloroform purification. DNA from FFPE tumors was isolated according to the S. DeVries/F. Waldman protocol[6] with minor modifications. Briefly, two 30-μm sections were obtained from FFPE tumors, treated with xylene, incubated in glycine-Tris-EDTA [100 mmol/L glycine, 10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA] and sodium sulfocyanate (1 mol/L), and finally digested with proteinase K and purified with phenol chloroform. All sections were previously examined and dissected with a scalpel to ensure at least 70% content of tumor cells.

BAC array platform. CGH was done onto the "1-Mb array" platform developed at the University of Pennsylvania (25) and previously used in similar studies (20, 22). Briefly, the platform is composed of a set of 4,134 publicly available human BAC clones spaced at ~1-Mb resolution across the genome. BAC DNA was amplified using oligonucleotide-primed PCR primers. At least two replicates of each BAC clone were printed on each slide using a Lucidea Array Spotter (Amersham Biosciences) and a spotting solution of 50% DMSO (25).

Array comparative genomic hybridization. For hybridization, 1 μg of test DNA and 1 μg of sex-matched pooled normal human DNA (obtained from a set of 10 healthy female or male volunteers) were labeled with either Cy3-dCTP or Cy5-dCTP by random priming (BioPrime Labeling kit, Invitrogen). The differentially labeled DNA samples were pooled, mixed with 100 μg of human COT-1 DNA, dried down, and rehydrated in 50 μL of a formamide-based buffer (25). After denaturation (10 min at 75°C) and preannealing (30 min at 37°C), hybridization was allowed for 48 to 72 h at 37°C in a moist chamber on a slowly rocking platform followed by a series of posthybridization washes: 2× SSC and 0.1% SDS (15 min, room temperature), 2× SSC and 50% formamide, pH 7.0 (15 min, 45°C), 2× SSC and 0.1% SDS (30 min, 45°C), and 0.2× SSC (15 min, room temperature). Finally, arrays were scanned on a GenePix 4000B dual scanner (Axon Instruments). Both test and reference DNA were labeled with the opposite dye in a separate experiment ("dye swap") to account for differences in dye incorporation and provide additional data points for analysis.

Data analysis. Fluorescence data from hybridization images were processed and analyzed with GenePix Pro 5.0 (Axon Instruments) to obtain the $\log_2$ ratios (tumor/reference) of each slide. aCGH normalization was done using the DNMAD application (26), which also allowed us to merge and filter replicate clones on the same slide and in the dye-swap experiment. We filtered out inconsistent replicates (those with a $\log_2$ ratio distance to the median $\log_2$ ratio of the replicates >0.3) and those clones that did not have available data in >70% of the cases (65 of 93 samples).

Finally, we established categorized copy number values using the binary segmentation algorithm implemented in the InSilico CGH software (0, 1, or -1 indicating no change, gain, or loss, respectively; ref. 27). We defined genomic regions as a group of at least two consecutive clones showing the same categorized copy number value. High-level DNA amplifications were considered when segmentation level was four times the segmentation gain level.

For visualization purposes, we used CGHAnalyzer (25, 28) and CGHExplorer (29) softwares.

Standardization of an artifactual-copy number variation pattern. We observed a recurrent genomic pattern of artifactual aberrations in our breast cancer sample set (see Results), which was previously reported in normal samples analyzed by cCGH (30) and recently also observed by aCGH and named artifactual-copy number variation (Ar-CNV).[7] Briefly, this artifactual pattern generates abnormal ratios in certain chromosomal regions, such as 1p36, 2q37, 4p16, 6p21, 9q34, 11q13, and 12q13 (Fig. 1A), which can be erroneously interpreted as gains in the analysis. We also obtained this Ar-CNV pattern in a set of normal FFPE tissue DNA versus normal control DNA hybridizations. Kirchhoff et al. (30) previously described a nonrandom pattern of deviations in normal samples by cCGH and, subsequently, applied a standardization approach to increase the specificity and the sensitivity of the technique, obtaining a dramatic decrease in false-positive results. Therefore, to diminish the effect of the Ar-CNV in our set, a standardization approach was applied to every sample showing this artifactual pattern. The standardization was done by subtracting from each clone $\log_2$ ratio the median value for that clone in the set of normal FFPE tissue DNA versus normal control DNA hybridizations that also presented the Ar-CNV pattern. After the standardization, cases were analyzed again and the aCGH profiles did not show the artifactual pattern anymore (Fig. 1B). Log_2 ratios of each sample are shown in Supplementary Table S2.

Fluorescence in situ hybridization studies. To verify the standardization approach, we did a fluorescence *in situ* hybridization (FISH)

[6] Protocol available from http://cc.ucsf.edu/people/waldman/Protocols/paraffin.html.

[7] D. Blesa et al. Detection of a pattern of artifactual copy number variations that can induce to overestimate changes on genome profiling analysis, submitted for publication.

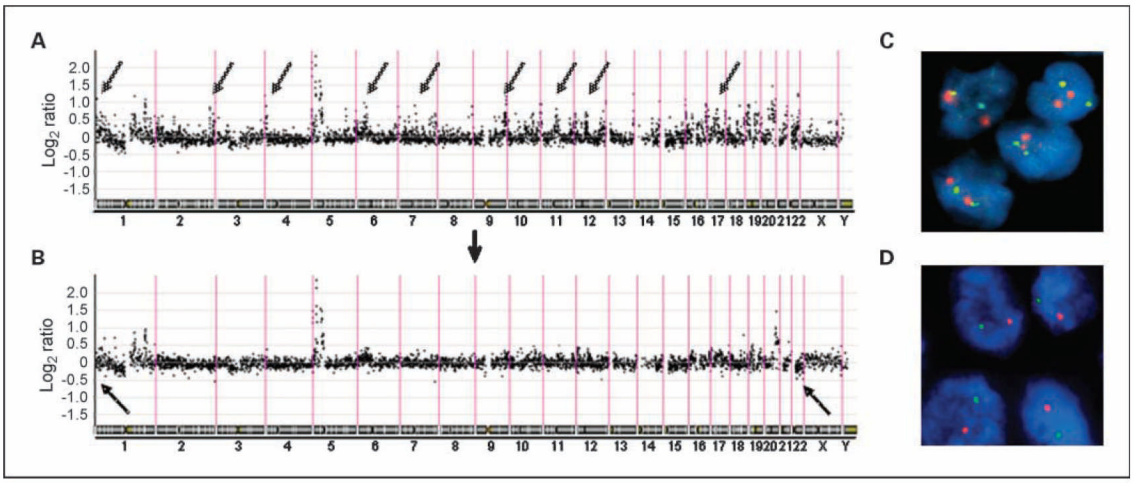

Fig. 1. Standardization of the Ar-CNV pattern. *A,* aCGH profile of a familial breast tumor sample that shows artifactual aberrations in the regions indicated by the arrows. *B,* aCGH profile of the same case after standardization. Aberrations at 1q, 5p, or 20q are considered gains because they are preserved after standardization. Arrows below the tumor profile indicate regions further validated by FISH analysis. *C,* FISH study using the 1p36 probe, showing two green signals (1p36 loci) and two red signals (centromeres). These data support the standardized aCGH pattern and not the nonstandardized one in which the gain was artifactual. *D,* FISH experiment with the chromosome 22 probe clearly shows a monosomy (one green and one red signal), supporting again the loss observed in the standardized profile.

analysis on tissue microarrays that contained the FFPE samples; their characteristics have been previously published (23, 24). We checked the copy number status of two different locations affected by Ar-CNV: 1p36 and 22q11-q12. The 1p36 probe was made of three BAC clones from the distal p-arm region of chromosome 1 (RP11-82D16, RP4-713A8, and RP4-740C4, located at 2.07, 2.25, and 2.30 Mb from the 1p telomere, respectively). These BACs were all labeled with dUTP-SpectrumGreen (Vysis, Inc.). The 1p36 probe also included a chromosome 1 centromeric probe "CEP1α Satellite DNA Spectrum-Orange" from Vysis. The 22q11-q12 probe consisted of two clones mapping at 22q11.21 (RP11-316L10 and RP11-330P17) labeled with dUTP-SpectrumGreen and three clones located on 22q12.2 (RP1-76B20, RP1-151I23, and RP3-394A18) labeled with dUTP-Spectrum-Orange. FISH analysis was done according to Vysis' instructions, with slight modifications. An average of 110 (50-200) well-defined nuclei was analyzed and the number of single copy gene and centromeric signals was scored.

Statistical analysis of aCGH data. We used a nonparametric Mann-Whitney *U* test to compare the mean number of genomic alterations among the four patient groups. The Statistical Package for the Social Sciences for Windows statistical software (SPSS, Inc.) was used for these comparisons. For the analysis of differences in the aberration frequency of specific chromosomal regions, we used the Stat POMELO tool[8] (31), applied Fisher's exact test, and adjusted *P* values for multiple testing using the false discovery rate approach (a *P* value of <0.05 was considered significant). Finally, hierarchical unsupervised clustering was done using correlation methods included in the Cluster software (32).

Results

Standardization procedure to diminish the effect of the Ar-CNV pattern. We analyzed tumor DNA from a total of 93 breast tumors (19 BRCA1, 24 BRCA2, 31 BRCAX, and 19 sporadic samples) using aCGH. We found a specific pattern of aberrations that was present in a high percentage of cases (78%). These genomic aberrations were coincident with the so-called Ar-CNV, described in a recent aCGH technical report. In this article, an aCGH pattern of specific Ar-CNV, observed in normal samples and distinct tumor types by using different array platforms and reproduced in several laboratories, is described.[7] We applied a standardization approach similar to the one used by Kirchhoff et al. (30) in their original cCGH studies to avoid an analogous issue (see Materials and Methods). An example of our standardization approach is shown in Fig. 1A and B. Some of the regions affected by Ar-CNV were further validated by FISH analysis (Fig. 1C and D; Supplementary Table S3). A closer correlation aCGH-FISH data was clearly found after standardization, showing a dramatic increase in confirmed aberrations (9% before to 82% after standardization). This analysis confirmed the utility of this correction.

Overall genomic changes in breast tumor classes. We determined the genome instability according to the number of CNVs (measured as the total number of gains, losses, and numerical aberrations) present in a tumor. *BRCA1*-associated tumors had the most unstable genome with a total of 28.0 ± 2.9 CNV, *BRCA2*-related tumors had 19.8 ± 2.3 CNV, BRCAX tumors showed 15.3 ± 1.9 CNV, whereas sporadic tumors presented 18.7 ± 1.9 CNV. These differences were statistically significant ($P < 0.05$, Mann-Whitney *U* test) when *BRCA1*-associated tumors were compared with the other tumor types (Supplementary Fig. S1A). No significant differences were found when a comparison between different types of recurrent mutations was made (data not shown).

Frequencies of genomic alterations. The frequency and distribution of genomic gains and losses of each group is shown in Fig. 2. Four genomic regions were altered in >40% of cases in all tumor groups: gains of 1q and 16p13.3 and losses of 8ptel-p12 and 16q. No specific aberrations were associated significantly with an exclusive breast tumor class. However, there were

[8] Statistics software available from http://pomelo.bioinfo.cnio.es.

some statistically significant differences in the pairwise comparisons (Table 1). Given that BRCAX tumors showed the lowest overall alteration frequency, many regions in BRCAX tumors were significantly different when compared with the other tumor classes (Table 1). Differences in the alteration frequencies in chromosome X were not considered because the reference for the sporadic breast cancer set was not sex matched.

There were aberrant regions at a high frequency (>50%) in each tumor class (Fig. 2). In summary, losses of 4q32.3-qtel, 5q, 13q, and 18q were more frequent in *BRCA1*-associated tumors. *BRCA2*-associated tumors presented recurrent gains of 8q12.3-qtel and losses of 11q23.1-qtel, 13q12.3-q21.33, and chromosome 22. Sporadic breast cancers had frequent gains of 8q13.1-qtel and losses of 4q24-qtel and 13cen-q31.3. Finally, BRCAX tumors did not show any highly recurrent aberration in addition to the commonly altered regions.

About high-level DNA amplifications, 8q24.11 and 8q24.12-q24.21 were the only two regions with a frequency >15% in all groups. No significant differences in high-level DNA amplification frequencies were found across tumor types. However, different tumor classes showed frequent high-level amplification (>15%) at distinct locations, such as 8p, 8q, 10p, 11q13, 12p13, and 13q34 (Table 1), pointing at subtle differences in the amplification targets of each tumor class. We looked in detail at specific regions of amplification that cover some known oncogenes, such as 8q24.21 (*c-MYC*) and 17q12 (*ERBB2*). *c-MYC* is amplified in all groups (37% BRCA1, 33% BRCA2, 26% sporadic, and 13% BRCAX). *ERBB2* was only amplified in 12.9% of BRCAX and 5.3% of sporadic cancers. Noteworthy, no high-level amplification of this gene was observed in *BRCA1*- and *BRCA2*-associated tumors.

Testing of the previously reported discriminative regions. To test the value of the discriminative regions described by Jönsson et al. (20), we used a hierarchical unsupervised clustering using the same discriminative chromosomal regions. We only included the mutation-positive BRCA carriers and sporadic breast cancer cases because these were the tumor classes previously used to describe these regions. We could distinguish two subgroups: one mainly composed of *BRCA1*-associated tumors (orange cluster) and other one mainly composed of *BRCA2*-associated and sporadic tumors (green cluster; Fig. 3A). However, we found BRCA2 (4) and sporadic (6) outlier tumors located in the BRCA1 subgroup and some BRCA1 samples (7) in the other branch. Jönsson et al. also found some outliers in their clustering. The authors suggested that ER status and grade might explain those outliers. When looking in detail at the ER status in our tumor series, we found that more than half the outliers in the BRCA1 branch (two BRCA2 and five sporadic tumors) were ER^-. Similarly, six of the seven BRCA1 outliers in the BRCA2 branch were ER^+ (Fig. 3A). This might indicate that these regions are mainly differentiating positive and negative ER tumors rather than BRCA mutation status.

Because Jönsson et al. only included in their study ER^- *BRCA1*-associated, ER^+ *BRCA2*-associated, and a mixture of $ER^{-/+}$ sporadic tumors, and we had a mixture of $ER^{-/+}$ in all tumor groups, we removed from our series all those cases that did not match the features of the series studied by Jönsson et al. (ER^+ *BRCA1*-associated and ER^- *BRCA2*-associated tumors). Without these tumors, we obtained a different cluster. The clustering analysis discriminated two groups, one of them (orange) mostly composed of ER^- tumors (17 ER^- and 2 ER^+ tumors) and a second branch (green) composed mainly of ER^+ tumors (22 ER^+ and 3 ER^- tumors; Fig. 3B). This time, the number of BRCA class outliers was reduced from 11 outliers (7 BRCA1 and 4 BRCA2; Fig. 3A) to 3 outliers (1 BRCA1 and 2 BRCA2; Fig. 3B). This cluster resembled the results obtained by Jönsson et al. These findings may highlight the importance of ER status in tumor development.

ER profiling of breast cancer. To further test the role of the ER, we divided all cases according to ER status, accounted for CNV in each group, and created genomic alteration frequency plots. The higher genomic instability in the ER^- tumors was remarkable, showing significant differences ($P < 0.05$) in CNV compared with the ER^+ tumor group (Supplementary Fig. S1B). We also saw this difference when we subdivided each tumor class according to ER status (Supplementary Fig. S1C).

Frequency plots of genomic alterations in the tumor groups according to the ER status are shown in Fig. 4A. Both groups of

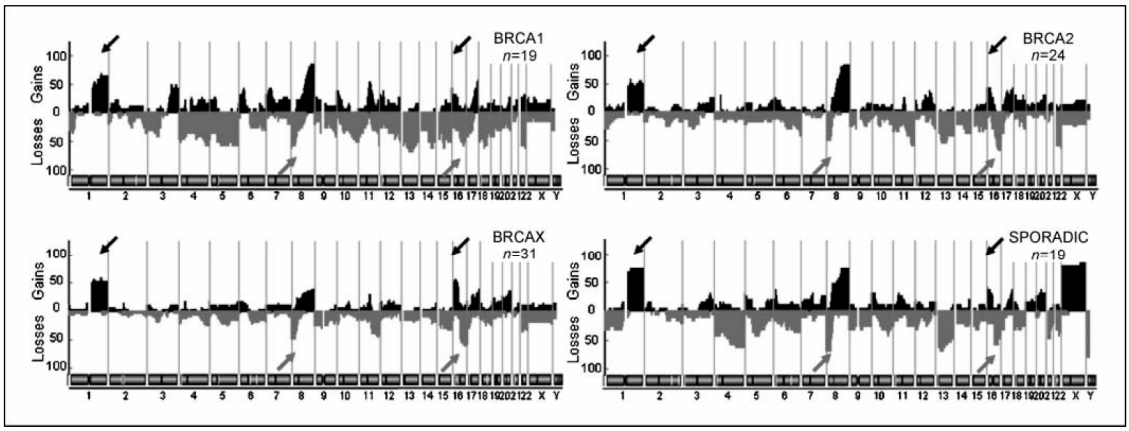

Fig. 2. Overall genomic aberration frequencies in BRCA1, BRCA2, BRCAX, and sporadic breast cancer samples. Black, frequency of gains; gray, frequency of losses. Arrows at chromosomes 1, 8, and 16 highlight the regions commonly altered in >40% of all breast cancer groups. Black arrows, common gains; gray arrows, common losses.

Table 1. Significant genomic aberrations (adjusted $P < 0.05$) and their frequencies among distinct breast tumor groups and recurrent high-level DNA amplifications

Chromosomal region	Frequencies of alteration (%)				Two-sided adjusted Fisher's exact *P*					
	BRCA1	BRCA2	BRCAX	Sporadic	B1vsB2	B1vsBX	B1vsS	B2vsBX	B2vsS	BXvsS
Gain										
8q22.1	**58**	**79**	32	**74**				*0.023*		
8q23.1-8q23.3	**74**	**83**	35	**74**				*0.010*		
8q23.3-8q24.13	**79**	**83**	35	**74**				*0.010*		
8q24.13-8qtel	**84**	**83**	39	**74**		0.077		*0.025*		
11q14.1	47	0	10	10	*0.005*					
Loss										
4q23	32	12	6	53						*0.020*
4q24	37	12	6	**58**						*0.005*
4q25	47	12	6	**58**		0.074				*0.005*
4q26-4q28.1	42	12	6	**58**						*0.005*
4q28.2	42	16	9	**58**						*0.031*
4q28.3	42	16	10	**63**						*0.022*
4q31.1	42	8	13	**63**					*0.009*	*0.022*
4q31.21	37	8	16	**63**					*0.009*	
4q32.1-4q32.2	**47**	12	23	63					*0.044*	
4q32.3	**52**	12	23	63					*0.044*	
4q33-4q34.1	**58**	12	26	63					*0.044*	
4q34.2-4q35.1	**58**	12	23	63					*0.044*	
4q35.1-4q35.2	**52**	12	26	63					*0.044*	
13q12.3	47	**50**	16	**68**						**0.013**
13q13.1-13q13.3	**53**	**50**	16	**68**						**0.013**
13q13.3-13q14.11	**58**	**54**	16	**68**						**0.013**
13q14.11-13q14.3	**63**	**54**	16	**68**		0.097				**0.013**
13q14.3-13q21.33	**68**	**54**	16	**58**		*0.019*				
13q22.1-13q22.2	**68**	42	16	**58**		*0.019*				
13q22.2-13q22.3	**68**	42	19	**58**		*0.048*				
13q31.1	**63**	42	19	**53**		*0.026*				
21q21.3	11	0	3	42					*0.025*	*0.046*
High-level DNA amplifications										
8p12-8p11.23	11	8	6	**16**						
8p11.22-8pcen	11	4	3	**16**						
8q22.1-8q22.3	5	**17**	10	**16**						
8q23.1-8q23.3	11	**21**	10	**21**						
8q24.11	**32**	**37**	**16**	**21**						
8q24.12-8q24.21	**37**	**37**	**16**	**21**						
8q24.21-8q24.22	**37**	**29**	13	**26**						
8q24.23-8qtel	**21**	**25**	10	**26**						
10ptel-10p15.3	0	0	0	**16**						
10p15.2	11	0	0	**16**						
10p15.1	0	0	0	**16**						
10p14	5	0	0	**16**						
11q13.3-11q13.4	11	12	**16**	0						
12p13.32-12p13.31	**16**	0	0	5						
13q34-13qtel	**16**	4	0	11						

NOTE: Frequency aberrations >50% are in bold. Entries in italics show $P < 0.05$. In addition, those high-level DNA amplifications with a frequency >15% (in bold) in at least one tumor group are shown.

tumors have common high frequent gains of 1q and 8q22.1-qtel and losses of 8ptel-p12 and 16q, which are similar to those described in all breast tumor classes. However, a set of genomic aberrations, such as -3p25.3-p21.3, -3p21.1-p14.2, +3q25.1-q26.1, -4q31.2-qtel, -5q, +10p14-p12.3, -12q14-q15, and -12q23.1-q23.31, presented significantly higher frequencies in ER^- tumors (Fig. 4B). It is noteworthy that 6q25.1 (the locus of the *ESR* gene) is more frequently deleted in ER^+ than in ER^- tumors.

In addition, ER^- tumors presented a trend toward having more high-level DNA amplifications than ER^+ tumors. There were also slight differences in the amplification target sites. For example, 8q24.21 (*c-MYC*) was amplified in 32% of the ER^- tumors and only in 20% of the ER^+ tumors. Moreover, 17q12 (*ERBB2*) was exclusively amplified in ER^- tumors (5%), whereas amplification at 20q13.12-q13.33 was only found in ER^+ tumors (8%). However, the only region with an amplification rate that was significantly different (adjusted *P* value < 0.05) comparing both groups was 13q34, which was only present in ER^- tumors (16%).

Discussion

The present aCGH-based study characterizes in detail the patterns of genomic alteration of the familial breast tumor classes (BRCA1, BRCA2, and BRCAX) and of sporadic breast

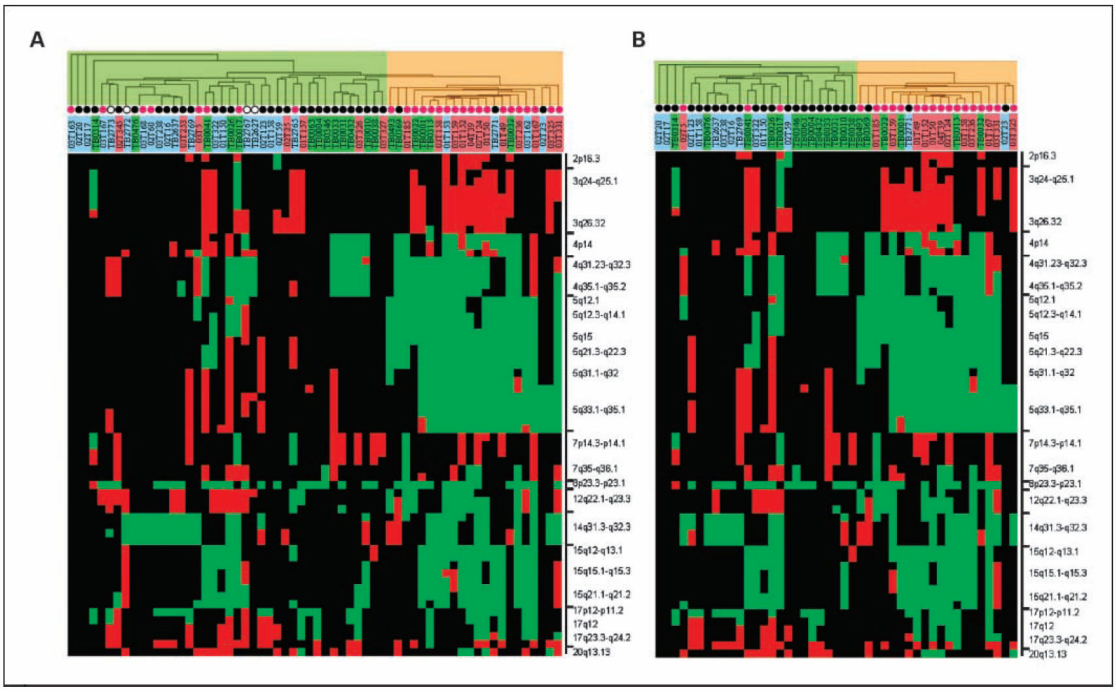

Fig. 3. *A,* unsupervised clustering of all BRCA1 (*red*), BRCA2 (*blue*), and sporadic (*green*) breast tumors of our collection using the regions reported as discriminative in the aCGH classifier by Jönsson et al. (20). Regions are listed according to chromosome number and are considered as gains (*red*), no change (*black*), or losses (*green*). Black circle, ER^+; red circle, ER^-; white circle, nonevaluated cases. *B,* unsupervised clustering of BRCA1 (*red*), BRCA2 (*blue*), and sporadic (*green*) breast tumors with BRCA1 ER^+ and BRCA2 ER^- tumors removed.

cancer, supports previous findings obtained using the cCGH technique and the same aCGH platform (20), and tests the discriminative regions defined in the latter study. To our knowledge, this is the first aCGH profiling of BRCAX-associated breast cancer samples and the second one of *BRCA1*- and *BRCA2*-associated tumors. Our results highlight the importance of ER status, suggesting that this feature should be considered when designing comparative studies of familial and sporadic breast cancer.

Genomic instability in familial and sporadic breast cancer. We have confirmed the high genomic instability in *BRCA1/2*-associated tumors that was previously observed in several articles using cCGH (13–16) and aCGH (20). The highest number of CNV was present in breast cancers associated with *BRCA1*, those related to *BRCA2* mutations were the second most unstable class of breast tumors, whereas sporadic cases showed more CNV than BRCAX-related breast cancers but less than those associated with *BRCA2* mutations (Supplementary Fig. S1A).

We distinguished common aberrations across all breast cancer groups, such as gains of 1q and 16p13.3 and losses of 8ptel-p12 and 16q, which are concordant with previous analyses by cCGH (15, 16, 33). These commonly altered regions could represent a set of shared aberrations that include important genes and characterize the breast cancer development in general.

Beyond these shared alterations, there were no specific genomic changes associated with any specific tumor class, although some recurrent aberrations were observed (Fig. 2). Thus, *BRCA1*-associated tumors were characterized by a high frequency of gains of 3q and 8q21.3-qtel and losses of 4q32.3-qtel and distinct regions of chromosome 5. *BRCA1*-associated tumors also had a significantly recurrent gain at 11q14.1 when compared with *BRCA2*-associated tumors (Fig. 1; Table 1). Some of these changes have been reported as discriminative in tumor class comparisons (13, 15, 20). On the other hand, *BRCA2*-associated tumors had frequent gains of 8q12.3-qtel (significant when compared with BRCAX) and losses of 11q23.1-qtel, 13q12.3-q21.33, and chromosome 22 (Fig. 1; Table 1). Gain of 17q22-q24 and losses of chromosome 13 among others have been reported as frequent changes in *BRCA2*-associated tumors (13, 20). Here, we found both changes at a high frequency, and we were also able to refine both changes to 17q23-qtel and 13q12.3-q21.33. BRCAX tumors were characterized by the lowest overall frequency of genomic alterations, presumably due to the high number of grade 1 samples in the BRCAX-associated breast cancer set (14 of 31; see Supplementary Table S1). Finally, regions recurrently aberrant in sporadic samples were concordant with the ones previously reported (34, 35).

In addition, amplification target sites slightly differed between tumor classes (Table 1). Interestingly, whereas c-*MYC* is amplified in all groups suggesting an universal amplification target site, neither *BRCA1*- nor *BRCA2*-associated tumors presented amplification at the *ERBB2* locus, as previously

described (10, 20). *ERBB2* amplification seems to be a marker that can help in discriminating familial samples, in which mutation analysis of the BRCA genes is advisable, saving time and effort.

With these results, the aCGH patterns of familial breast cancer may assist in the diagnostics because there are differences between BRCA1 and BRCA2/BRCAX patients. However, our findings show some differences when compared with previous studies about the discriminative regions associated with familial and sporadic cancer. These variations are largely attributable to differences in sample size or the type of *BRCA1/2* mutation of the set of familial cancer samples, but we propose an additional role of ER status. Whereas previous studies mainly studied BRCA1/ER$^-$ and BRCA2/ER$^+$ tumors, we were able to study a set of *BRCA1*- and *BRCA2*-associated breast cancers that contained both ER$^+$ and ER$^-$ tumors.

ER status modulates the genomic changes in the tumor. Steroid receptor status is one of the main differentiating features of sporadic breast cancer, as has been shown in gene expression studies (36) and in aCGH profiles (34, 35, 37). With regard to familial breast cancer, *BRCA1*-associated tumors mainly are associated with ER negativity, and for this reason, most of studies on BRCA1 tumors only include ER$^-$ *BRCA1*-associated breast cancer samples. Examples are the study by Hedenfalk et al. (38) using expression arrays and more recently the study by Jönsson et al. (20) using aCGH. It has been suggested that the ER status may have confounded the findings in these studies (39, 40). When we examined our series using Jönsson's discriminative regions, we found one cluster mainly composed of *BRCA1*-associated cases and another one consisting of *BRCA2*-associated and sporadic cases, although many outliers were present (Fig. 3A). The reason could be that

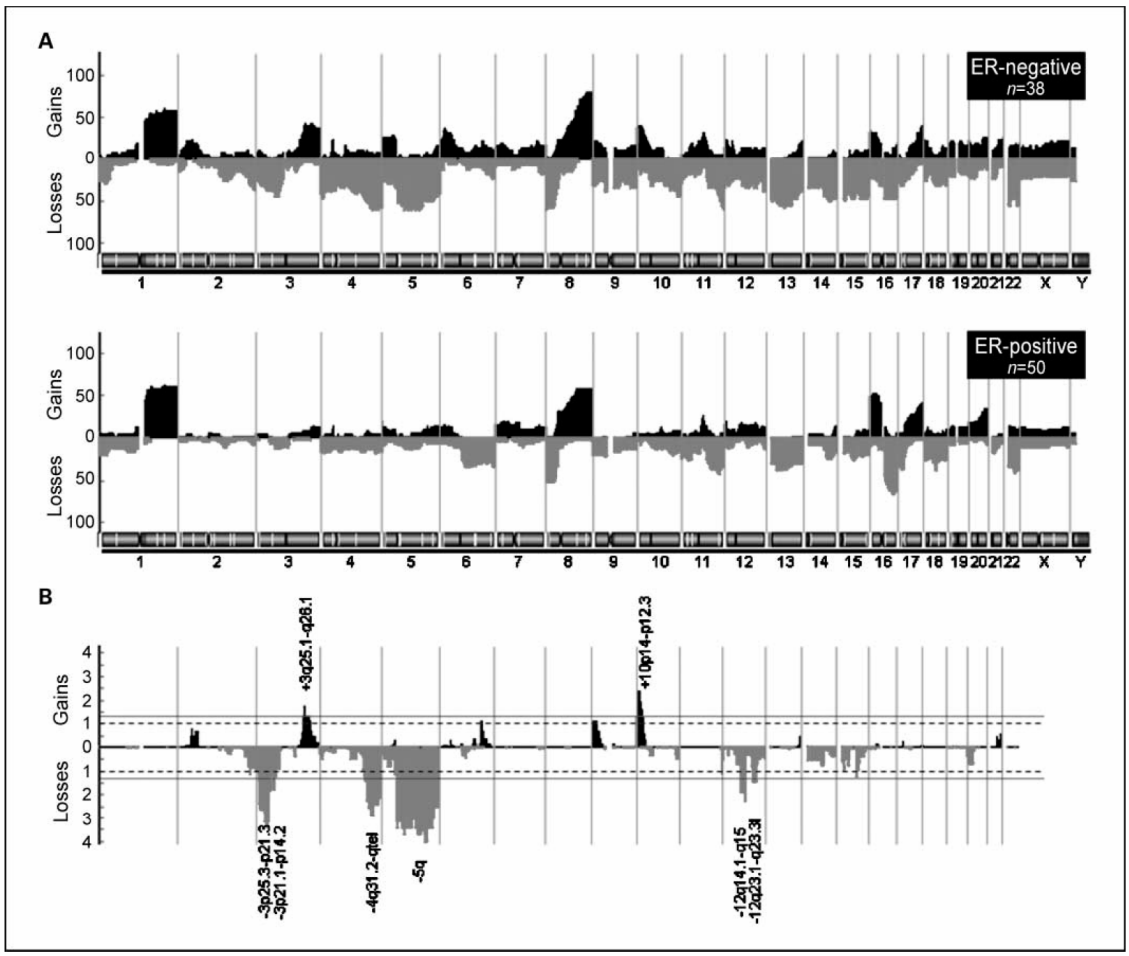

Fig. 4. *A*, overall genomic aberration frequencies of all tumors classified according to ER status. Black, frequency of gains; gray, frequency of losses. *B*, representation of the negative log of the adjusted *P* values, obtained from Fisher's exact test when comparing the gain (*top*) and loss (*bottom*) frequencies among ER$^-$ versus ER$^+$ tumors. Dashed lines, adjusted *P* value = 0.1 [-log (adjusted *P*) = 1]; gray lines, adjusted *P* value = 0.05 [-log (adjusted *P*) = 1.3]. Those aberrations that are statistically significant (*P* = 0.05, Fisher's exact test) are shown.

Jönsson's discriminative regions were found only comparing BRCA1 ER$^-$, BRCA2 ER$^+$, and sporadic tumors with either status, whereas our series included BRCA1 and BRCA2 tumors with a mix of ER$^-$ and ER$^+$ status. In fact, after removing BRCA1 ER$^+$ and BRCA2 ER$^-$ tumors from our series, we obtained one cluster composed of ER$^-$ tumors (BRCA1 and sporadic) and a second cluster mainly formed by ER$^+$ tumors (BRCA2 and sporadic), the number of BRCA outliers being clearly reduced (Fig. 3B). Both ER clusters present obvious genomic differences from each other. These results suggest that ER status is an important marker of changes in the tumor genome, independent of the underlying mutation status.

When we grouped all tumors, including BRCAX samples, according to their ER status, the higher genomic instability of ER$^-$ tumors was clearly shown not only when all cases were grouped together (Supplementary Fig. S1B) but also within each group defined by BRCA mutation status (Supplementary Fig. S1C). Several genomic aberrations were recurrently present in ER$^-$ tumors, such as -3p25.3-p21.3, -3p21.1-p14.2, +3q25.1-q26.1, -4q31.2-qtel, -5q, +10p14-p12.3, -12q14-q15, and -12q23.1-q23.31, which significantly discriminated between both ER tumor groups (Fig. 4B). Most of these aberrations are concordant with those previously reported in aCGH studies of sporadic breast cancer (34, 35, 37), and some were also included in Jönsson's discriminative regions (20). In fact, losses of loci at 4q and 5q were reported as frequent in *BRCA1*-related breast cancers but also in ER$^-$ *BRCA2*-associated breast tumors and ER$^-$ sporadic samples (41, 42). On the other hand, ER$^+$ tumors showed a lower level of genomic instability and a trend to present alterations at chromosome 16 (+16p and -16q), which are classic features of ER$^+$ and low-grade tumors (35, 43, 44). Interestingly, absence of expression of ER seems to be due to a mechanism independent of copy number losses at the *ESR* locus, given that ER$^+$ tumors presented a higher deletion frequency at this locus than ER$^-$ tumors (Fig. 4A). About high-level DNA amplifications, ER$^-$ tumors had higher frequencies of these aberrations than ER$^+$ tumors. We propose here the significantly recurrent amplification at 13q34 in ER$^-$ tumors as a candidate aberration for further characterization. The same differences between both tumor groups (ER$^+$/ER$^-$) about genomic instability and aberrant regions were also present when tumors were subdivided by BRCA mutation status (Supplementary Fig. S2), which emphasizes the role of the ER status independent of the breast tumor class. In this sense, comparisons between tumor classes with the same ER status could elucidate aberrations specifically associated with a breast tumor class. However, a larger amount of samples would be needed.

In summary, we present a genomic characterization of familial and sporadic breast cancer samples using aCGH. We confirm the higher genomic instability of BRCA1/2 tumors and describe the common existence of aberrations that could represent the set of genomic abnormalities characteristic in breast cancer development. We also report a set of altered regions that discriminate between tumor groups but are not specific for only one tumor class. Our findings suggest a critical role for the ER status as a marker of the genomic changes present in a tumor. The patterns of genomic changes among familial and sporadic breast cancers are strikingly similar, and differences are mainly determined by the ER status rather than by the BRCA mutation status as is generally accepted. Thus, ER negativity might allow or involve a set of genomic alterations different from those in tumors expressing ER. These aberrant regions may contain interesting genes that determine the higher aggressiveness of the ER$^-$ tumors and, hence, should be analyzed in detail, whereas the BRCA gene mutation status (mainly BRCA1) would contribute to the genomic profile of abnormalities by increasing or modulating the genomic instability.

Acknowledgments

We thank the Spanish National Tumor Bank Network and Luz Álvarez and Miguel Urioste (Familial Cancer Unit) for the collection of the samples, the Immunohistological Unit and Carmen Martin (Molecular Cytogenetics Group, Spanish National Cancer Center) for their technical support, and Angelica C. Medina (Medical Genetics Division, University of Pennsylvania) for her technical assistance.

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Oncogene (2007), 1–11

www.nature.com/onc

ORIGINAL ARTICLE

Distinct genomic aberration patterns are found in familial breast cancer associated with different immunohistochemical subtypes

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Five breast cancer subtypes have been described in sporadic breast cancer (SBC) using expression arrays: basal-like, ERBB2, normal breast-like, luminal A and B. These molecular subtypes show different genomic aberration patterns (GAPs). Recently, our group described these breast cancer subtypes in 50 non-*BRCA1/2* familial tumors using immunohistochemistry assays. We extended this study to the other classes of familial breast cancer (FBC), including 62 tumors (18 *BRCA1*, 16 *BRCA2* and 28 non-*BRCA1/2*), with the same panel of 25 immunohistochemical (IHC) markers and histological grade obtaining a similar classification. We combined these data with results generated by a 1 Mb BAC array-based CGH study to evaluate the genomic aberrations of each group. We found that *BRCA1*-related tumors are preferentially basal-like, whereas non-*BRCA1/2* familial tumors are mainly luminal A subtype. We described distinct GAPs related to each IHC subtype. Basal tumors had a greater number of gains/losses, while luminal B tumors had more high-level DNA amplifications. Our data are similar to those obtained in SBC studies, highlighting the existence of distinct genetic pathways of tumor evolution, common to both SBC and FBC.
Oncogene advance online publication, 10 December 2007; doi:10.1038/sj.onc.1210975

Keywords: hereditary breast cancer; BRCA1; BRCA2; BRCAX; array-CGH; breast cancer subtypes

Introduction

Human breast cancer is a heterogeneous disease encompassing different pathological entities and a range of clinical behavior. Studies in sporadic breast cancer (SBC) based on expression profiling reflect this heterogeneity (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003; Sotiriou *et al*., 2003). Five distinct SBC subtypes defined by different expression patterns and clinical outcomes have been reported: luminal A and B, ERBB2, basal-like and normal breast-like (Sorlie *et al*., 2003). Recently, two different studies have described distinct spectra of DNA copy number alterations associated with each SBC subtype. A higher number of gains/losses were associated with basal-like tumors, while high-level DNA amplification was more frequent in luminal-B subtype tumors (Bergamaschi *et al*., 2006; Chin *et al*., 2006).

Familial breast cancer (FBC) includes tumors from patients carrying mutations in the two known breast cancer susceptibility genes: *BRCA1* (Miki *et al*., 1994) and *BRCA2* (Wooster *et al*., 1995). However, most of FBC patients do not carry mutations in these genes, and are known as non-*BRCA1/2* or BRCAX cases. *BRCA1*-associated tumors can be differentiated from *BRCA2*, BRCAX and SBC based on their immunohistochemical (IHC) profiles (see reviews (Honrado *et al*., 2005a; Lacroix and Leclercq, 2005)). Most reports suggest that *BRCA1*-associated tumors have a basal-like phenotype because they share many expression, IHC and clinical features with basal-like cancers (see reviews (Tischkowitz and Foulkes, 2006; Turner and Reis-Filho, 2006)). On the other hand, *BRCA2*-associated tumors are only distinguished from BRCAX and SBC by the expression of DNA repair proteins such as RAD51 and CHEK2 (Honrado *et al*., 2005b). The association of *BRCA2*-associated tumors with luminal A subtype has been suggested in a single expression study (Sorlie *et al*., 2003). Finally, recent IHC studies have underlined the heterogeneous of the BRCAX tumors, which resembles the one obtained in SBC studies (Oldenburg *et al*., 2006; Honrado *et al*., 2007).

Although the genomic characterization of *BRCA1*-, *BRCA2*- and BRCAX-associated tumors has been reported in different studies (Tirkkonen *et al*., 1997; Alvarez *et al*., 2005; Jonsson *et al*., 2005; Melchor *et al*., in press; van Beers *et al*., 2005), the possible correlation between the molecular subtypes of FBC and the

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Received 11 September 2007; revised 1 November 2007; accepted 8 November 2007

genomic aberrations has not been clarified yet. In the present study, we have classified 62 FBC samples by IHC analysis into five subgroups. We have demonstrated a correlation between IHC subtypes and specific Genomic Aberration Patterns (GAPs). Our results are very similar to those obtained previously in SBC, and support that breast cancer arises from several distinct biological mechanisms, which are common to both FBC and SBC.

Results

Data from 62 FBC samples (18 *BRCA1*-, 16 *BRCA2*- and 28 BRCAX-associated tumors) previously studied by IHC and aCGH have been correlated to establish their IHC subtypes and the pattern of genomic aberrations.

Unsupervised cluster analysis

We performed an unsupervised hierarchical cluster analysis with 25 IHC markers (Supplementary Table 1) and histological grade. The 62 tumor samples were classified into two main groups, associated with their estrogen receptor (ER) status. The ER-negative branch (Figure 1, left) included 20 tumors of grade 2 and 3 (brown and red squares, respectively), hormonal receptors negativity and overexpression of proteins that promote cell cycle progression and cell proliferation. The ER-positive branch involved 42 tumors mainly of grade 1 (green squares) or 2, hormonal receptors positivity and expression of proteins related to the inhibition of the cyclin-CDK complexes, and luminal epithelial proteins (cytokeratin 8 (CK8)) (Figure 1, right).

The ER-negative branch can be split into two subgroups: one characterized by ERBB2 overexpression (4 tumors), and a second subgroup (16 tumors) defined by overexpression of basal markers, such as, cytokeratin 5 (CK5), vimentin, survivin and epidermal growth factor receptor (EGFR). The subgroup associated with ERBB2 overexpression was composed exclusively of BRCAX tumors, while the basal-like subgroup included mainly *BRCA1*-associated tumors (11 of 16 tumors).

Three subgroups were found within the ER-positive branch. One of the subgroups (brown branch in Figure 1) contained 16 tumors that are mostly grade 1, and overexpress hormonal receptors, CK8, BCL2 and proteins that inhibit cell cycle progression (for example, p27 and p16). Of note, most of these tumors were BRCAX samples (13 of 16). A total of 17 tumors composed the second subgroup (purple branch in Figure 1). These samples were characterized by a higher grade than the previous group, low or lack of expression of hormonal receptors as compared with the other groups and overexpression of other proteins such as cyclin A (related to the cell cycle progression) and TOPOII (related to cell growth). A mixture of the three FBC groups was found in this subgroup (Figure 1). We termed these two subgroups as luminal A and B, respectively, according to the parameters (such as grade and differential expression of TOPOII) that have been used previously to discriminate between them (Sorlie *et al*., 2001; Sotiriou *et al*., 2003). Finally, a group of nine tumors were described (Figure 1, green branch), which had luminal features (positive for hormonal receptors and CK8), high grade and overexpression of CHEK2 and survivin. This group was composed mainly of *BRCA2*-associated tumors (5 of 9 samples). We called this group as 'unclassified' tumors, since the features did not match with those previously established for 'normal breast-like' or the other tumor groups.

A summary of the FBC distribution in the different IHC subtypes is shown in Table 1. Most of *BRCA1*-associated tumors show a basal-like phenotype, while

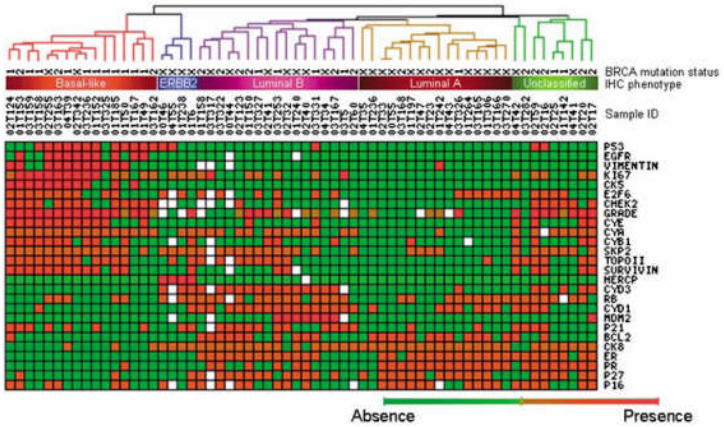

Figure 1 Unsupervised hierarchical clustering of 62 FBC samples. White squares correspond to data not available. The percentage of positive cells for each immunohistochemical marker is represented as a range of color between the most green (lowest percentage) and the most red (highest percentage). Intermediate colors represent percentages between the lowest and the highest.

Table 1 Immunophenotype distribution of the FBC tumors based on the 25 IHC markers and histological grade

	Basal-like	*ERBB2*	*Luminal B*	*Luminal A*	*Unclassified*
BRCA1 ($n=18$)	**11 (61.1%)**[a]	0	4 (22.2%)	1 (5.6%)	2 (11.1%)
BRCA2 ($n=16$)	3 (18.8%)	0	**6 (37.5%)**	2 (12.5%)	**5 (31.2%)**
BRCAX ($n=28$)	2 (7.1%)	4 (14.3%)	7 (25.0%)	**13 (46.5%)**[b]	2 (7.1%)
Total ($n=62$)	16 (25.8%)	4 (6.5%)	17 (27.4%)	16 (25.8%)	9 (14.5%)

Abbreviations: FBC, familial breast cancer; IHC, immunohistochemical. Bold values indicate the BRCA-class that presents the highest percentage of a concrete immunophenotype. [a]Significant differences in the Fisher's exact test ($P \leqslant 0.05$) when comparing basal-like distribution of *BRCA1* versus *BRCA2*, and *BRCA1* versus BRCAX. [b]Significant differences in the Fisher's exact test ($P \leqslant 0.05$) when comparing luminal A distribution of BRCAX versus *BRCA1* and BRCAX versus *BRCA2*.

BRCA2-associated tumors are mainly found in the luminal B or unclassified subtypes. The ERBB2 subtype was composed entirely of BRCAX samples, although most of BRCAX malignancies had a luminal A phenotype. IHC characteristics of the different subtypes and statistical comparisons are shown in Table 2.

Genomic characterization of each FBC subtype

Classifying the FBC tumors in five subtypes according to the IHC clusters, we collected the array-CGH data of these samples (Melchor *et al*., in press), and assessed their copy number of genomic aberrations (CNA). Basal breast tumors had the highest mean of genomic changes (30.75 ± 3.0 CNA), while luminal A tumors had the lowest mean number of CNA (10.87 ± 1.9). Luminal B, ERBB2 and unclassified tumors had 20.00 ± 2.7, 14.75 ± 6.2 and 14.44 ± 3.7 CNA, respectively (Figure 2). Differences in the amount of CNA were statistically significant ($P<0.05$, Mann–Whitney *U*-test) when comparing basal breast tumors with any of the other cancer subtypes.

Next, we plotted the GAPs of each FBC subtype (Figure 3). The most recurrent aberrations (frequency over 50%) in luminal A subtype were gains at 1q and 16p, and losses at 11q23 and 16q. Luminal B tumors exhibited as recurrent aberrations (over 50%): −8ptel-p12, +8q21-qtel, −11q23.3-qtel, −14q31, +16p, −16q, +20q13.13-qtel, −22q. Given that the ERBB2 subtype was composed of only four tumors, the GAP was not informative, though all cases had gain at 17q12 (*ERBB2* locus). Basal breast tumors clearly presented an unstable GAP with many aberrations with a frequency over 50%, such as, −3p21-p13, +3q25.1-q26, −4p, −4q22.1-qtel, −5q, −8ptel-p12, +8q22.1-qtel, among others. Finally, unclassified tumors showed an intermediate level of genome instability as compared to luminal A and B tumors. The only two genomic aberrations with a frequency higher than 50% in unclassified tumors were gains at 1q32.2 and 8q21.12-qtel. When we compared the aberration frequencies between the subtypes as determined by IHC, significant differences were only found when compared basal versus non-basal breast tumors (Supplementary Figure 1).

High-level DNA amplifications were more frequently found in luminal B, basal-like and ERBB2 tumors than in luminal A cancers ($P=0.021$, 0.036 and 0.042, respectively) (Figure 4a). Some regions of high-level DNA amplification tend to be subtype specific such as 20q13 in luminal B tumors, 6p22 and 13q34 in basal-like, and as expected 17q12 in ERBB2 tumors (Figure 4b).

Discussion

We have shown that FBC can be grouped in the different breast cancer subtypes described previously in SBC using IHC (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003; Sotiriou *et al*., 2003). In addition, we have performed a complete genomic characterization of the subtypes. Differences in the levels of genomic instability, GAPs, and high-level DNA amplification target regions were found associated with FBC subtypes.

Common breast cancer heterogeneity and association of BRCA *status with breast cancer subtype*

We have previously shown the heterogeneity present in BRCAX and SBC tumors using 25 IHC markers and grade (Honrado *et al*., 2007). In the current work, we extended the study to the other classes of FBC, including 62 cancers (18 *BRCA1*-, 16 *BRCA2*- and 28 BRCAX-associated), and found similar results: five FBC subtypes were established using unsupervised cluster analysis of IHC of multiple proteins and grade (Figure 1). Each FBC subtype was associated with its own characteristic IHC features (Table 2). These subtypes were similar to those obtained in SBC using expression analysis: basal-like, ERBB2, luminal A and B (Perou *et al*., 2000; Sorlie *et al*., 2001). Although we were not able to distinguish a normal breast-like subtype, we identified a fifth group with intermediate characteristics between luminal A and B subtypes, which we titled the 'unclassified group'. Given its higher grade and over-expression of proliferation markers (cyclins, Ki-67 and so on), this subtype could be more aggressive than the luminal A subtype and thus, it could be analogous to the luminal C subtype named by Sorlie *et al*. (2001) or luminal-like 3 described by Sotiriou *et al*. (2003). These findings emphasize the existence of different breast tumor subtypes that represent distinct biological entities not only in SBC, but also in FBC.

Table 2 Immunohistochemical markers and statistical comparisons between the different IHC subtypes

	Basal-like (N = 16)	*ERBB2* (N = 4)	*Luminal B* (N = 17)	*Luminal A* (N = 16)	*Unclassified* (N = 9)	P[1]	P[2]	P[3]	P[4]	P[5]
HERCP(+3)										
1–2+	16 (100.0)	0	16 (100.0)	16 (100.0)	9 (100.0)	NS	0.000	NS	NS	NS
3+	0	4 (100.0)	0	0	0					
ER										
Negative	16 (100.0)	4 (100.0)	4 (23.5)	3 (18.8)	1 (11.1)	0.000	0.037	0.047	0.019	0.033
Positive	0	0	13 (76.5)	13 (81.3)	8 (88.9)					
PR										
Negative	15 (93.8)	4 (100.0)	11 (64.7)	2 (12.5)	1 (11.1)	0.000	NS	NS	0.000	0.009
Positive	1 (6.2)	0	6 (35.3)	14 (87.5)	8 (88.9)					
P53										
Negative	4 (25.0)	2 (50.0)	16 (94.1)	16 (100.0)	7 (77.8)	0.000	NS	0.025	0.003	NS
Positive	12 (75.0)	2 (50.0)	1 (5.9)	0	2 (22.2)					
BCL2										
Negative	15 (93.8)	4 (100.0)	7 (41.2)	4 (25.0)	7 (77.8)	0.001	NS	0.086	0.002	NS
Positive	1 (6.3)	0	10 (58.8)	12 (75.0)	2 (22.2)					
Ki-67										
0–4%	2 (12.5)	1 (25.0)	10 (58.8)	16 (100.0)	4 (44.4)	0.000*	0.105*	NS*	0.000*	0.102*
5–24%	5 (31.3)	3 (75.0)	5 (29.4)	0	5 (55.6)					
25–100%	9 (56.3)	0	2 (11.8)	0	0					
EGFR										
Negative	6 (37.5)	4 (100.0)	15 (100.0)	16 (100.0)	9 (100.0)	0.000	NS	0.054	0.050	NS
Positive	10 (62.5)	0	0	0	0					
CK5										
Negative	5 (31.3)	4 (100.0)	17 (100.0)	16 (100.0)	9 (100.0)	0.000	NS	0.026	0.052	NS
Positive	11 (68.8)	0	0	0	0					
Vimentin										
Negative	5 (31.3)	4 (100.0)	14 (100.0)	15 (93.8)	9 (100.0)	0.000	NS	0.052	NS	NS
Positive	11 (68.8)	0	0	1 (6.3)	0					
Grade										
1	0	1 (50.0)	4 (28.6)	10 (71.4)	1 (11.1)	0.001*	NS*	0.066*	0.000*	NS*
2	2 (12.5)	0	6 (42.9)	3 (21.4)	1 (11.1)					
3	14 (87.5)	1 (50.0)	4 (28.6)	1 (7.1)	7 (77.8)					
CK8										
Negative	13 (81.3)	0	2 (11.8)	0	2 (22.2)	0.000	NS	NS	0.003	NS
Positive	3 (18.8)	4 (100.0)	15 (88.2)	16 (100.0)	7 (77.8)					
Cyclin D1										
Negative	15 (93.8)	3 (75.0)	1 (5.9)	9 (56.3)	5 (55.6)	0.000	NS	0.000	NS	NS
Positive	1 (6.3)	1 (25.0)	16 (94.1)	7 (43.8)	4 (44.4)					
Cyclin D3										
Negative	16 (100.0)	0	1 (6.7)	15 (93.8)	4 (44.4)	0.000	0.054	0.000	0.002	NS
Positive	0	3 (100.0)	14 (93.3)	1 (6.3)	5 (55.6)					
Cyclin E										
Negative	4 (25.0)	4 (100.0)	15 (88.2)	16 (100.0)	5 (55.6)	0.000	NS	NS	0.003	NS
Positive	12 (75.0)	0	2 (11.8)	0	4 (44.4)					
Cyclin A										
Negative	0	2 (50.0)	4 (23.5)	15 (93.8)	1 (12.5)	0.000	NS	NS	0.000	NS
Positive	16 (100.0)	2 (50.0)	13 (76.5)	1 (6.3)	7 (87.5)					
Cyclin B1										
Negative	6 (37.5)	2 (66.7)	13 (81.3)	14 (93.3)	5 (55.6)	0.004	NS	NS	0.023	NS
Positive	10 (62.5)	1 (33.3)	3 (18.8)	1 (6.7)	4 (44.4)					
P27										
Negative	14 (87.5)	3 (75.0)	4 (25.0)	4 (25.0)	3 (33.3)	0.000	NS	0.079	0.079	NS
Positive	2 (12.5)	1 (25.0)	12 (75.0)	12 (75.0)	6 (66.7)					

Table 2 (*Continued*)

	Basal-like (N = *16*)	*ERBB2* (N = *4*)	*Luminal B* (N = *17*)	*Luminal A* (N = *16*)	*Unclassified* (N = *9*)	P^1	P^2	P^3	P^4	P^5
SKP2										
Negative	2 (12.5)	0	4 (25.0)	12 (75.0)	2 (22.2)	0.063	NS	NS	0.000	NS
Positive	14 (87.5)	4 (100.0)	12 (75.0)	4 (25.0)	7 (77.8)					
P16										
Negative	10 (62.5)	1 (33.3)	7 (50.0)	5 (33.3)	3 (33.3)	NS	NS	NS	NS	NS
Positive	6 (37.5)	2 (66.7)	7 (50.0)	10 (66.7)	6 (66.7)					
P21										
Negative	9 (56.3)	2 (50.0)	5 (31.3)	14 (87.5)	5 (55.6)	NS	NS	0.019	0.007	NS
Positive	7 (43.8)	2 (50.0)	11 (68.8)	2 (12.5)	4 (44.4)					
RB										
Negative	11 (68.8)	0	3 (18.8)	7 (43.8)	1 (12.5)	0.005	NS	NS	NS	NS
Positive	5 (31.3)	3 (100.0)	13 (81.3)	9 (56.3)	7 (87.5)					
MDM2										
Negative	16 (100.0)	3 (100.0)	8 (53.3)	16 (100.0)	8 (88.9)	0.093	NS	0.000	0.093	NS
Positive	0	0	7 (46.7)	0	1 (11.1)					
E2F6										
Negative	6 (37.5)	1 (33.3)	9 (60.0)	10 (62.5)	4 (44.4)	NS	NS	NS	NS	NS
Positive	10 (62.5)	2 (66.7)	6 (40.0)	6 (37.5)	5 (55.6)					
TOPOII										
Negative	4 (25.0)	2 (50.0)	7 (46.7)	15 (93.8)	3 (33.3)	0.019	NS	NS	0.000	NS
Positive	12 (75.0)	2 (50.0)	8 (53.3)	1 (6.3)	6 (66.7)					
CHEK2										
Negative	3 (18.8)	2 (100.0)	11 (84.6)	16 (100.0)	3 (33.3)	0.000	NS	0.101	0.000	0.066
Positive	13 (81.3)	0	2 (15.4)	0	6 (66.7)					
Survivin										
Negative	4 (25.0)	3 (75.0)	13 (86.7)	16 (100.0)	4 (44.4)	0.000	NS	0.067	0.001	NS
Positive	12 (75.0)	1 (25.0)	2 (13.3)	0	5 (55.6)					

Abbreviations: EFGR, epidermal growth factor receptor; ER, estrogen receptor; IHC, immunohistochemical; NS, not significant; PR, progesterone receptor. *P*-values from the Fisher's exact test performed when compared one IHC subtype versus the rest: P^1, basal-like versus nonbasal like tumors; P^2, ERBB2 versus nonERBB2 tumors; P^3, luminal B versus nonluminal B tumors; P^4, luminal A versus nonluminal A tumors; P^5, unclassified versus non-unclassified tumors. (*) χ^2-test was used for Ki-67 and grade. *P*-values$\leqslant$0.10 are represented; *P*-values$\leqslant$0.05 were considered as significant; NS, not-significant *P*-values.

The proportion of these subtypes in FBC is not the same as in SBC (Sorlie *et al*., 2001) or BRCAX samples (Oldenburg *et al*., 2006; Honrado *et al*., 2007). In the present study, 26% of FBC had a basal-like IHC phenotype compared to 15% in SBC and BRCAX (Sorlie *et al*., 2001; Oldenburg *et al*., 2006) (Table 1). This difference can be attributable to the presence of *BRCA1*-associated tumors in our series, which are prone to have a basal phenotype (Sorlie *et al*., 2003; Tischkowitz and Foulkes, 2006; Turner and Reis-Filho, 2006). In our sample set, 61% of *BRCA1*-associated tumors comprised a significant proportion of the basal-like IHC phenotype. Nineteen percent of *BRCA2*-associated tumors, and 7% of BRCAX samples were also found to be basal-like (Table 1). All BRCAX cancers were studied previously for hypermethylation of the *BRCA1* gene promoter and loss of heterozygosity; interestingly, the BRCAX samples that had basal-like phenotype showed biallelic inactivation of the *BRCA1* gene (Honrado *et al*., 2007). This model of carcinogenesis in the BRCAX tumors is in agreement with the low level of *BRCA1* mRNA expression reported in basal-like cancers (Staff *et al*., 2003; Wei *et al*., 2005; Turner *et al*., 2006). On the other hand, the ERBB2 subtype comprised only of BRCAX tumors (four cases, ~14%) (Table 1). This finding is in concordance with the low incidence of *ERBB2* amplification in *BRCA1/2* mutation carriers described before (Grushko *et al*., 2002; Lakhani *et al*., 2002; Palacios *et al*., 2003; Adem *et al*., 2004). A significant association was found between most of BRCAX samples (45%) and luminal A phenotype, as seen in previous analyses (Oldenburg *et al*., 2006; Honrado *et al*., 2007). Finally, *BRCA2*- and non-basal *BRCA1*-associated tumors were mainly related to the luminal B phenotype (37 and 22%, respectively). *BRCA2*-associated tumors also comprised an important proportion of the unclassified samples (Table 1). In contrast, Sorlie *et al*. (2003) linked *BRCA2*-associated

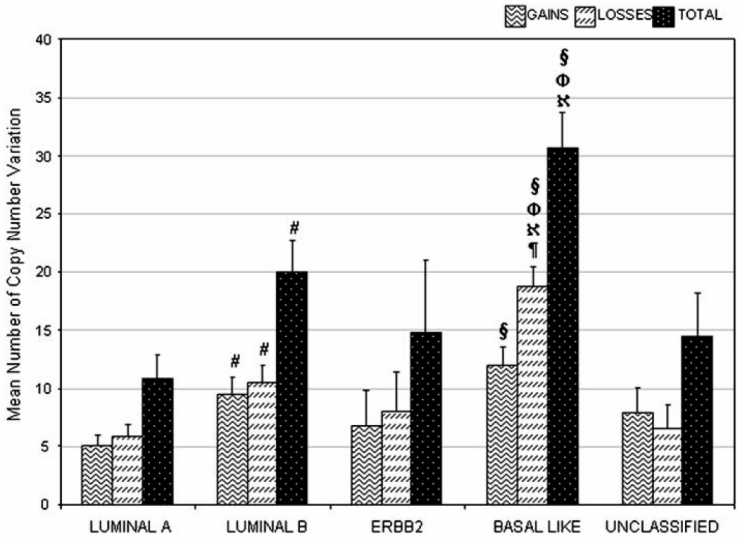

Figure 2 Mean number of genomic alterations. Significant differences (*P*-value <0.05 in Mann–Whitney *U*-Test) are shown in different characters depending on the comparison: basal-like versus luminal A (§), luminal B (Φ), unclassified (ϰ) ERBB2 (¶) and (#) luminal B versus luminal A tumors.

tumors to the luminal A subtype. This discrepancy may be caused by the small sample size given that the authors studied only two samples while we have a larger cohort of 16 *BRCA2*-associated cancers.

Distinct genomic aberration patterns associated with each FBC subtype

Different genomic characteristics have been recently associated with each of the five subtypes of SBC (Bergamaschi *et al*., 2006; Chin *et al*., 2006). We have studied the genomic change features of the five FBC subtypes using array-CGH data from a previous analysis (Melchor *et al*., in press). Basal-like tumors showed the highest genomic instability (Figure 2), consistent with two previous studies in SBC (Bergamaschi *et al*., 2006; Chin *et al*., 2006). In contrast, luminal A tumors had the lowest number of genomic aberrations. Bergamaschi *et al*. (2006) found the lowest number in ERBB2 tumors, but the low number of ERBB2 samples in our study (four cases) did not allow us to draw any significant conclusions.

The FBC subtypes defined by IHC exhibited distinct GAP (Figure 3). Basal-like tumors showed a specific GAP with many altered chromosomal sites such as −3p, +3q, −4p, −4q, −5q, −8p, +8q and so on. Some of these aberrations (for example, −3p25, −4p, −4q22-q35.1, −5q and so on) were significantly associated with basal-like tumors when compared with non-basal tumors (Supplementary Figure 1). The close association between basal-like phenotype and *BRCA1*-associated tumors explains the similarities that are found when comparing the GAP from basal-like tumors and *BRCA1*-associated tumors (Jonsson *et al*., 2005). Luminal A tumors frequently have +1q, +16p, −11q23 and −16q; luminal B tumors show genomic aberrations of other regions such as −8p, +8q, +20q and −22q. These subtype-GAPs and the recurrent chromosomal aberrations within each subtype are in agreement with those previously reported in SBC (Supplementary Table 2) (Bergamaschi *et al*., 2006; Chin *et al*., 2006).

Differences in high-level DNA amplifications

High-level DNA amplifications were found more frequently in luminal B tumors than in the other tumor subtypes (Figure 4a). In addition, the regions targeted for amplification differed slightly between the IHC subtypes (Figure 4b). As expected, ERBB2 tumors had a frequent amplification at 17q12 and overexpression of ERBB2. Approximately 20% of the luminal B breast cancer subtype had amplification of regions, such as, 8p11-p12, 8q24, 11q13.3-q13.4, 17q25 and 20q13. Luminal A cancers had few high-level amplifications with the exception of frequent amplification at 11q13 (*CCND1* locus), which was also found in luminal B tumors. This finding could explain the CCND1 over-expression present in the luminal tumors (Figure 1), and it is in concordance with the studies that show a negative correlation between *CCND1* amplification and basal-like phenotype (Reis-Filho *et al*., 2006; Elsheikh *et al*., 2007). Finally, basal-like tumors have high-level DNA amplification frequently at 8q24, 12p13 and 13q34

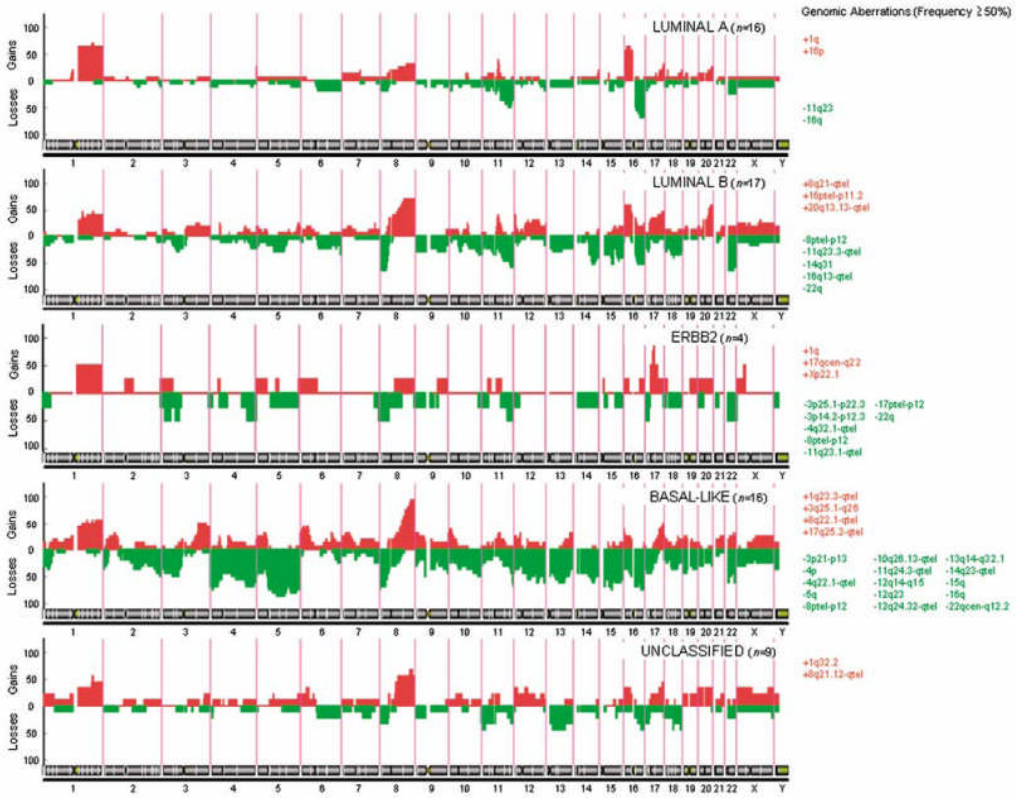

Figure 3 Genomic aberration patterns among the different IHC breast cancer subtypes. Red and green indicate frequencies of gains and losses, respectively. Genomic aberrations with a frequency over 50% in each IHC subtype are shown.

(Figure 4b). Most of these amplification sites are similar to the regions described in SBC subtypes (Bergamaschi *et al*., 2006; Chin *et al*., 2006) (Supplementary Table 2). Amplification of 11q13 appears recurrent in luminal cancers, whereas amplifications of 8p11-12 and 20q13 are more frequently found in the luminal B subtype, and amplification of 17q21 in the ERBB2 subtype. These common amplifications found in both FBC and SBC subtypes could represent targets for therapy, as has already been established with *ERBB2* and trastuzumab. A high frequency of amplification at 8q24 (*MYC* locus) was described in our basal-like FBC, an aberration less common in basal-like SBC (Chin *et al*., 2006; Rodriguez-Pinilla *et al*., 2006). Because Al-Kuraya *et al*. (2004) reported a greater frequency of *MYC* amplification in medullary carcinomas, a specific subtype of basal-like tumors that is very frequent in *BRCA1*-associated cancers (Lakhani *et al*., 1998), we checked whether that difference could be due to the presence of medullary carcinomas in our basal-like FBC. However, *MYC* amplification was present in 3 of 6 medullary FBC and 4 of 10 non-medullary basal-like FBC in our set (data not shown). A larger series of cases will be necessary to confirm or to rule out this association.

Conclusions

Our findings demonstrate that breast cancer can be subdivided into distinct subtypes independently whether the tumors are familial or sporadic. The FBC subtypes differed in terms of tumor histology, IHC portraits and genomic changes patterns (Table 3). A higher prevalence of basal-like phenotype is present in *BRCA1*-related tumors, while luminal A phenotype is recurrent in *BRCAX*-associated cancers. In addition, basal-like malignancies had more gains and losses than the other subtypes, while luminal B cancers showed more high-level DNA amplifications. These characteristics are similar to those recently described in SBC (Bergamaschi *et al*., 2006; Chin *et al*., 2006). These findings support the existence of distinct genetic pathways of tumor evolution, common to sporadic and FBC, which underlie the

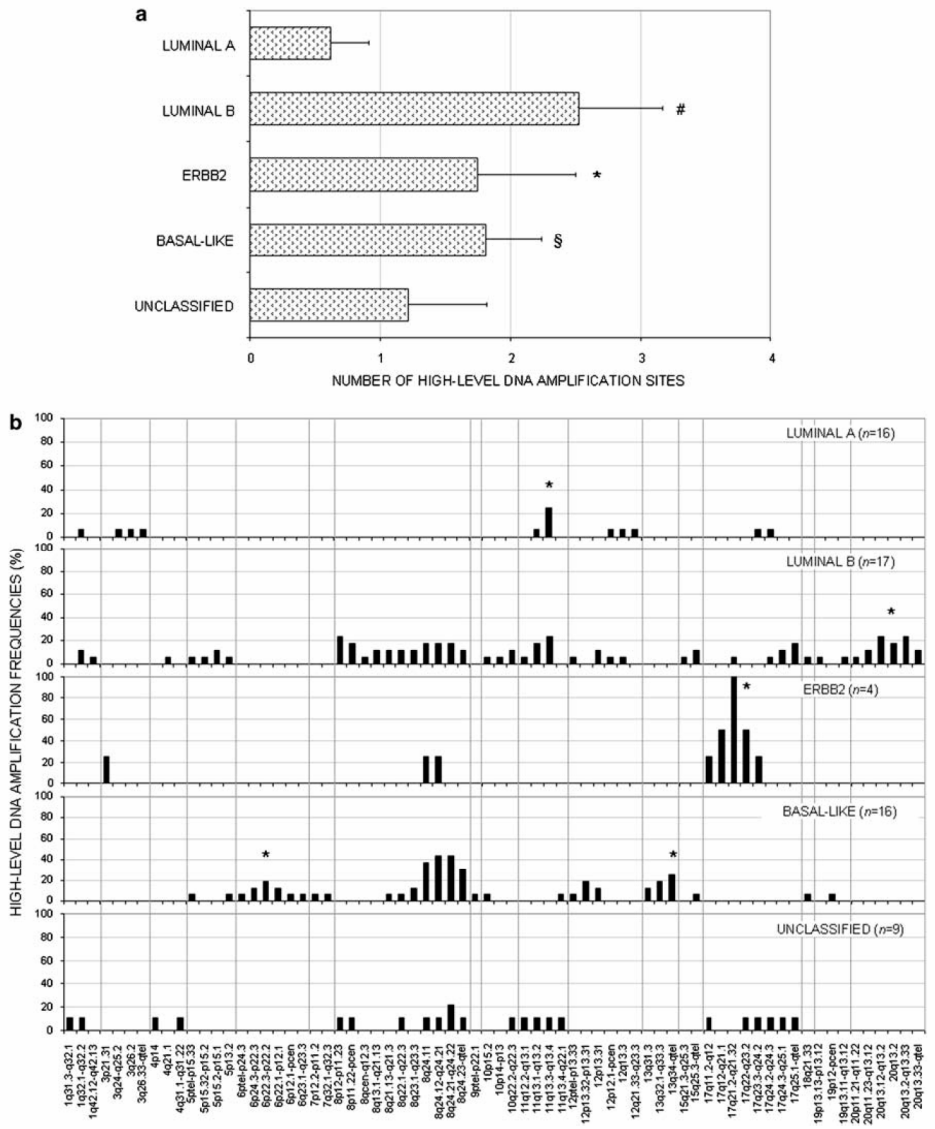

Figure 4 (**a**) Mean number of high-level DNA amplifications in each IHC breast cancer subtype. Significant differences (*P*-value <0.05 in Mann–Whitney *U*-Test) are shown in different characters depending on the comparison: luminal A versus luminal B (#), ERBB2 (*) and basal-like (§). (**b**) Frequency of high-level DNA amplification in each IHC breast cancer. Chromosomal sites of amplification are written in the *x* axis. Vertical gray lines separate chromosomes. Asterisks (*) point out specific amplification sites on each subtype.

pathogenesis of the different breast tumor subtypes and may explain their distinct biological behavior. Furthermore, we would postulate that gene-expression profiling, clinical presentation and response to therapy also differ in the five FBC IHC subtypes, as already reported in SBC subtypes (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003; Carey *et al*., 2006; Hu *et al*., 2006). Taking into account these differences, the BC subtypes should be studied as distinct entities to better describe their features, as has been done for basal-like tumors (Turner and Reis-Filho, 2006; Yehiely *et al*., 2006; Vincent-Salomon *et al*., 2007).

Table 3 Summary of features of IHC–FBC subtypes: basal-like, ERBB2, luminal A and B

	FBC class (%)							
IHC subtype	*BRCA1*	*BRCA2*	*BRCAX*	*Genomic instability*	*Recurrent gains*	*Recurrent losses*	*High-level DNA amplifications*	*Amplification targets*
Basal-like	68.75	18.75	12.50	High	1q, 3q, 8q, 17q	3p, 4p, 4q, 5q, 8p, 10q, 11q, 12q, 13q, 14q, 15q, 16q, 22q	Medium	6p22, 8q24, 13q34
ERBB2	0	0	100	Low	1q, 17q, Xp	3p, 4q, 8p, 11q	Medium	8q24, 17q12–q21
Luminal A	6.25	12.50	81.25	Low	1q, 16p	11q, 16q	Low	11q13
Luminal B	23.53	35.30	41.17	Medium	8q, 16p, 20q	8p, 11q, 14q, 16q, 22q	High	8p11–p12, 11q13, 20q13

Abbreviations: FBC, familial breast cancer; IHC, immunohistochemical. The first set of columns represents the distribution of every FBC class among one IHC phenotype. The second set of columns is the level of genomic instability (see Figure 2 for further details), with the recurrent gains and losses (see Figure 3 for further details of the chromosomal regions). The third set of columns shows the level of high-level DNA amplifications with the most recurrent targets (see Figure 4 for further details).

Materials and methods

Tumor samples and patients

We compiled 62 paraffin-embedded tumor tissues, which had been analysed previously both by IHC (Palacios *et al*., 2003, 2005; Honrado *et al*., 2005b) and aCGH (Melchor *et al*., in press). These breast cancer samples were selected from families with at least three women affected with breast and/or ovarian cancer and at least one of them diagnosed before 50 years of age, or from families with female breast and/or ovarian cancer and at least one case of male breast cancer. All cases were studied for mutations and large rearrangements in the *BRCA* genes using standard procedures (Osorio *et al*., 2000; Diez *et al*., 2003). A total of 18 cases had mutations in the *BRCA1* gene, 16 patients presented mutations in the *BRCA2* gene and 28 cases were negative for germ-line mutations in the *BRCA* genes (non-*BRCA1/2* or BRCAX).

Morphological evaluation, TMA construction and IHC studies

Histological sections from all 62 samples were reviewed by two pathologists (EH and JP). The Nottingham histological grading system was used to assess the grade of invasive ductal carcinomas.

Representative areas of the 62 tumors were carefully selected on H&E-stained sections and marked on individual paraffin blocks. Two tissue cores were obtained from each specimen and included in Tissue Micro-Arrays (TMA), whose characteristics have been previously published (Palacios *et al*., 2003, 2005; Honrado *et al*., 2005b).

Briefly, IHC assays were performed by the Envision method (Dako, Glostrup, Denmark) with a heat-induced antigen retrieval step. Sections from the tissue array were immersed in 10 mM boiling sodium citrate at pH 6.5 for 2 min in a pressure cooker. For the 25 antibodies used in this study, dilutions and suppliers are listed in Supplementary Table 1.

Between 150 and 200 cells per core were scored for the percentage of cells with positive nuclei or cytoplasm, depending upon the marker. We evaluated nuclear staining for ER, progesterone receptor, p53, Ki-67, cyclins D1, D3, E and A, p16, p27, p21, Skp2, retinoblastoma protein, E2F6, MDM2, topoisomerase IIα, survivin and CHEK2; evaluation of cytoplasmic staining was carried out for BCL2, vimentin, CK5/6, CK8 and cyclin B1, as described previously (Palacios *et al*., 2005). HER-2 expression was evaluated according to the four-category (0 to 3+) Dako system proposed for the evaluation of the HercepTest, and HER-2 expression of 3+ was the only value considered positive, as published earlier (Palacios *et al*., 2005).

Array comparative genomic hybridization analysis

Genomic DNA isolation from the 62 formalin-fixed paraffin-embedded (FFPE) tumors was performed as previously described (Melchor *et al*., in press). Briefly, two 30-μm sections were obtained from FFPE tumors, treated with xylene, incubated in Glycine Tris-EDTA (100 mM glycine, 10 mM Tris, pH 8.0, 1 mM EDTA) and NaSCN (1 M) and finally digested with proteinase K and purified with phenol chloroform. All sections were previously examined and dissected with a scalpel to ensure at least 70% content of tumor cells.

Comparative genomic hybridization was carried out onto the '1 Mb BAC' array platform developed at the University of Pennsylvania (Greshock *et al*., 2004). DNA probe labeling, aCGH protocol and array data analysis have been described previously (Melchor *et al*., in press). Briefly, in the array data analysis, aCGH normalization was carried using the DNMAD application (Vaquerizas *et al*., 2004). The normalized profiles were processed using the Binary Segmentation algorithm implemented in the Insilico CGH software (Vaquerizas *et al*., 2005). This algorithm defines genomic segments, which have an estimative copy number value in $\log_2$ ratio (that is, the median $\log_2$ ratio of the contained clones). Those segments with $\log_2$ ratio $\geqslant 0.1$ were considered as gains, while those with $\log_2$ ratio $\leqslant 0.1$ were categorized as losses. High-level DNA amplifications were considered when $\log_2$ ratio $\geqslant 0.4$.

Statistical analysis

Hierarchical unsupervised cluster analysis was performed by means of the UPGMA method using correlation distance and Euclidean distance between markers. The cluster was displayed using SOTAARRAY (Herrero *et al*., 2001) (software available at http://gepas.bioinfo.cipf.es). IHC results were represented by range of color from green to red, the lowest and the highest percentage of positive cells for each marker, respectively. Exceptions were grade that was scaled as 33% 'expressed' for grade 1 (green), 66% for grade 2 (brown) and 100% for grade 3 (red), and HER-2 that was scaled as 100% for positive (3+) (red) and 0% for negative (green) (Figure 1). Fisher's exact test was used to determine the differences in the expression of each marker between groups, except for Ki-67 and grade, which were calculated using χ^2-square test. The statistical software SPSS for Windows (SPSS Inc., Chicago, IL) was used for this analysis.

Regarding the array-CGH data, we used a non-parametric Mann–Whitney *U*-test to compare the mean number of genomic alterations among the different established groups. The SPSS software was used for these comparisons. For the analysis of differences in the aberration frequency of specific chromosomal regions, we used the Stat POMELO tool (http://pomelo.bioinfo.cnio.es) (Herrero *et al*., 2003), applied Fisher's

exact test and adjusted *P*-values for multiple testing using the FDR approach (a *P*-value <0.05 was considered significant).

Abbreviations

aCGH, array-based comparative genomic hybridization; CNA, copy number of genomic aberrations; ER, estrogen receptor; FBC, familial breast cancer; GAP, genomic aberration pattern; IHC, immunohistochemical; SBC, sporadic breast cancer.

Acknowledgements

We thank the Spanish National Tumor Bank Network, and Luz Álvarez and Miguel Urioste from the Familial Cancer Unit for the collection of the samples. For their technical assistance, we thank the Immunohistological Unit, and Carmen Martin and Juan C Cigudosa from the Molecular Cytogenetics Group, at the Spanish National Cancer Center and C Angelica Medina from Medical Genetics Division of the University of Pennsylvania. We also thank Susanna Leskelä for English assistance. *LM Grant sponsor*: Spanish Ministry of Education and Science FPU AP-2004-0448. Short stays sponsorships: ICRETT/05/063 and EMBO Short-Term Fellowship ASTF 162-05. MJG was supported by the Fundación Científica de la Asociación Española contra el Cáncer. This study has been partially funded by projects SAF03-02497 and SAF 06-06149 from the Spanish Ministry of Education and Science; and by a grant from Breast Cancer Research Foundation (BCRF) to KLN.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

Manuscript submitted for publication

Title: An Integrative Hypothesis about the Origin and Development of Sporadic and Familial Breast Cancer Subtypes

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ABSTRACT

Do breast cancer tumours have a common cell origin? Do different breast cancer molecular phenotypes arise from distinct cell types? The studies we have performed during the last few years in familial breast tumours (*BRCA1*, *BRCA2* and non-*BRCA1/2*) widen previous questions about sporadic breast cancer to hereditary breast cancer. Array-CGH studies show the existence of universal genomic aberrations in familial and sporadic breast cancer subtypes that may be required for the breast tumour development. *BRCA1* seems to play a crucial role in estrogen receptor (ER)-negative Cancer Stem Cells, driving the tumour development mostly towards a basal-like or, in some cases, a luminal B phenotype, but also other carcinogenetic events are proposed to explain the remaining tumour subtypes. The existence of common genomic alterations in basal-like, ERBB2 and luminal B breast tumours may suggest a common cell origin or clonal selection of these tumour subtypes, arising from an ER-negative Cancer Stem or Progenitor Cell. Finally, specific genomic aberrations in ER-positive tumours could represent imbalances that give rise to proliferation advantages when cells are exposed to estrogen. We propose a combination of the cancer stem cell hypothesis (for the carcinogenesis processes) and the clonal selection models (in terms of tumour development). We uphold that sporadic and familial tumour subtypes: basal-like, ERBB2, and luminal B, have an ER-negative Breast Stem/Progenitor Cell origin while luminal A tumours arise from an ER-positive Progenitor Cell, supporting an hierarchical breast carcinogenesis model, but crucial genomic imbalances are clonally selected in the tumour development.

INTRODUCTION

It is known that normal mammary tissue comprises different cell populations. The undifferentiated cohort of multipotent cells includes breast stem cells (SC), characterized by their capacity for self-renewal and differentiation into cell lineages, and progenitor breast cells, an amplifying population derived from SCs with limited lifespan and proliferation. At the end of these cell lineages, the differentiated cohort of breast cells involves myoepithelial, ductal epithelial, and alveolar cells. In normal development, mammary SCs give rise to two SCs (symmetric self-renewal), which produces SC expansion, or to one identical SC and a committed progenitor cell (PC), which undergoes cellular differentiation (asymmetric self-renewal) [1].

Breast cancer is initiated by carcinogenesis in a group of cells. The "stochastic model" of carcinogenesis proposes that first, malignant transformation occurs by multiple mutations in a random single cell, and second, there is a subsequent clonal selection. In contrast, the "hierarchical model" of carcinogenesis or "Cancer Stem Cell (CSC) Hypothesis" upholds that the malignant transformation occurs in subset of normal stem and progenitor cells, probably through de-regulation of self-renewal pathways [2]. The identification of human breast cancer initiating cells, which were able to generate breast tumours when injected in immuno-deficient NOD/SCID mice, strongly supports the unique ability of a set of cells to generate carcinogenesis in a proper cell niche [3].

Different breast cancer molecular subtypes with distinct clinical behavior have been described by expression array analyses in sporadic breast cancer: basal-like, ERBB2, luminal A and B, and normal breast-like [4,5]. According to the CSC hypothesis, the type of carcinogenetic event and the target cell are the underlying causes of the heterogeneity in breast cancer [6]. In this sense, carcinogenetic events in an ER-negative SC would give rise to basal-like, ERBB2, or luminal B breast cancer subtypes whereas in an ER-positive PC they would produce a luminal A phenotype [6,7].

Familial breast cancer arises from patients who have mutations in the two known breast cancer susceptibility genes (*BRCA1* or *BRCA2*) or in putative unknown genes that could explain the familial pattern of inheritance (non-*BRCA1/2* or BRCAX familial cases). We have seen the same molecular heterogeneity in familial and sporadic breast cancer by immunohistochemical (IHC) analysis (Table 1). This shared heterogeneity points out the existence of a common set of carcinogenetic pathways in both familial and sporadic breast cancer, where the *BRCA* genes could play a crucial role as proposed before [8].

HYPOTHESIS

Our studies in familial breast cancer using IHC and genomic changes profiling supports an integrative hypothesis of CSC and clonal selection models. We include the *BRCA* genes concept into the hierarchical model of carcinogenesis, extend the view to both familial and sporadic breast cancer, and use the genomic aberration pattern of breast tumour subtypes as a tool to elucidate the cancer cell origin and the clonal selection (Figure 1). We support the crucial role of *BRCA1* as a mammary SC regulator and that *BRCA1* silencing, in most of tumours, gives rise to basal-like carcinomas. We also propose biological

explanations for those *BRCA1*-associated cancers that do not display a basal-like phenotype: a) the *BRCA1* silencing occurs in a different CSC, or b) *BRCA1* mutation type may differ from those of basal-like carcinomas. The *ERBB2* amplification could be the earliest carcinogenetic event in an ER-negative SC/PC to give rise to the ERBB2 breast cancer subtype and it would not be compatible with mutations in the *BRCA1/2* genes. We think that luminal B tumours derive from ER-negative PCs that have crucial proliferation advantages, probably due to oncogene amplification and/or over-expression. Finally, we suggest that carcinogenesis in ER-positive PCs generate luminal A cancers. Data from genomic analyses showed common aberrations in: 1) all breast cancer subtypes, which could represent required genomic imbalances for the breast cancer development, 2) tumours that putatively derive from ER-negative SC/PCs (basal-like, ERBB2, and luminal B), which could support their common initial tumour environment, and 3) ER-positive tumours, which could reveal genomic imbalances that give proliferation advantage in that tumour cell niche.

SUPPORT FOR THE HYPOTHESIS

Breast cancer stem cells and *BRCA1*: the basal-like phenotype pathway

BRCA1-associated tumours are characterized by high grade, absence of expression of steroid receptors, ERBB2, and BCL2; and over-expression of basal cytokeratins (CK5/6), P53, and EGFR [9,10]. These features are also characteristic to basal-like carcinomas [4]. From a genomic perspective, the pattern of genomic aberrations in *BRCA1*-associated cancers and sporadic basal-like carcinomas are quite similar: gains at 3q and losses at 4p, 4q, 5q, 13q [11-16]. This is probably due because most of *BRCA1*-associated cancers were classified as basal-like carcinomas using expression profiling [17] and IHC analysis [18,19] (Table 1). Many studies have hypothesized about the close relationship among *BRCA1*-associated tumours and basal-like carcinomas [20,21]. In this line, it has been proposed that *BRCA1* acts as a SC regulator needed for luminal differentiation, thus its absence would generate the undifferentiated features of the basal-like phenotype [8].

Although most of *BRCA1*-associated tumours present a basal-like phenotype, this molecular subtype can also be found in sporadic, *BRCA2*- and BRCAX-associated cancers with a frequency of 10-15% (Table 1). In our studies, BRCAX-associated breast cancer with a basal-like subtype presented double inactivation of *BRCA1*: gene promoter hypermethylation and LOH [22]. In sporadic breast cancer, Turner and colleagues reported a frequent *BRCA1* promoter hypermethylation in metaplastic carcinomas, a specific subtype of basal-like carcinomas, and an overall low level of BRCA1 expression in basal-like cancers due to mechanisms different from *BRCA1* promoter hypermethylation such as high expression of ID4, a supressor of BRCA1 [23]. Our array-CGH study in familial breast cancer that was classified according to IHC subtypes revealed similar genomic aberrations in sporadic and familial basal-like carcinomas, the latter one composed mainly of *BRCA1*-associated tumours [24] (Figure 2). These findings corroborate the crucial role of *BRCA1* in the development of basal-like carcinomas (Figure 1A).

Recently, Yehiely and colleagues extended the role of BRCA1 as a SC regulator proposed earlier [8], explaining the transformation of a basal-like mammary SC to basal-like cancer cell [20]. The

carcinogenetic event in basal-like carcinomas is the LOH in *BRCA1* mutation carriers or the downregulation of BRCA1 in the other tumour classes (Figure 1A). This primary event could result in (Figure 1B): 1) the arrest of the luminal differentiation process, which determines the undifferentiated phenotype of basal-like carcinomas and thus its unique expression of basal cytokeratins (CK5/6), 2) an increment of the telomerase activity, since BRCA1 suppresses telomerase expression, and 3) DNA repair-defects that generate an inactivation or loss of ATM [25], and an increase in the genomic instability, which may be the reason for the greater number of genomic gains and losses in sporadic and familial basal-like cancers (Figure 2). In the tumour development, subsequent changes such as over-expression of cyclin E (to inactivate RB), EGFR (to activate PI3-Kinase pathway), TP53 (probably due to mutations in the gene) take place (Figure 1B). Data from expression and IHC analysis support this model since over-expression of cyclin E, EGFR, and TP53 are characteristic features both in basal-like carcinomas [26,27] and *BRCA1*-associated tumours [28-30]. Downregulation of PTEN may also occur in basal-like tumours because of specific recurrent genetic aberrations in its chromosomal region (10q23.3) [31].

However, the existence of subgroups recently described within the basal-like subtype and its controversy highlights the heterogeneity that still remains within this breast cancer subtype [32]. Although silencing of BRCA1 appears to be the key event in the basal-like carcinogenesis, one could expect a heterogeneous inactivation of *BRCA1* in basal-like cancers. It would be highly interesting to further study this breast cancer subtype.

The ERBB2 phenotype pathway: the incompatibility of amplification/over-expression and *BRCA1/2* mutation

Over-expression of ERBB2 occurs in 15-20% of sporadic and BRCAX-associated cancers [22], while it is very low or inexistent in *BRCA1/2*-associated tumours (0 to 3%) [29,30,33]. The correlation between over-expression and gene amplification has been demonstrated in many studies. In this sense, no FISH-amplification of *ERBB2* (in correlation with its absence or low expression) has been reported in *BRCA1*- or *BRCA2*-associated tumours [29,33,34].

Why do not *BRCA1/2*-associated cancers amplify and/or over-express *ERBB2*? Although there is a hypothesis about the physical co-deletion of *ERBB2* and *BRCA1* loci (as a second hit in *BRCA1*-mutation carriers) [35], this does not explain the lack of ERBB2 over-expression in *BRCA2*-associated cancers. Recently, we postulated that ERBB2 over-expression in cancer cells that have altered *BRCA1/2* genes do not give a survival advantage and, thus, do not proliferate [10]. Defects in the DNA repair system may not be compatible with the proliferation stress signal of the ERBB2 tyrosin kynase, and therefore, ERBB2 tumour cells that suffer significant genomic changes would not survive. The low genomic instability of ERBB2-related tumours when compared to other tumour subtypes supports this hypothesis [15,16,24].

The *ERBB2* amplification/over-expression has been proposed as a carcinogenetic event in ER-negative SC/PC [7] (Figure 1A). Given that *BRCA1* is not altered, the ERBB2 CSC undergoes luminal differentiation, expressing luminal cytokeratins (i.e. CK8/18) (Figure 1B)

The luminal B phenotype pathway: proliferation advantages

The luminal B breast cancer subtype is characterized by high/medium grade, variable expression of hormonal receptors, expression of luminal cytokeratins (CK8/18), and up-regulation of cell cycle (e.g. CCNE1) and cell growth (e.g. TOPO II) promoters [5]. Genomic analyses in luminal B sporadic and familial breast cancer subtypes showed more high-level DNA amplifications than in the other subtypes [15,16,24]. Another interesting feature of this cancer phenotype is its association with a poorer prognosis than luminal A tumours in terms of disease relapse [5]. This subtype has been found in a small proportion in all breast cancer classes (Table 1).

The CSC hypothesis proposed that luminal B tumours derive from primitive ER-negative CSC or PC, which could undergo luminal differentiation and display a variable expression of ER protein (Figure 1A-B) [6,7]. Patients who develop ER-positive tumours are frequently treated with hormonal therapy. However, this treatment would only produce transient remissions in luminal B patients, since the targets of carcinogenesis are ER-negative SCs [6]. This hypothesis is one possible explanation for the high disease relapse present in luminal B tumours. Nevertheless, there are questions that need to be addressed:

What determines a luminal B phenotype? From all those carcinogenetic events occurred in an ER-negative SC to become CSC, we think that the crucial one for the development of luminal B carcinomas is the amplification and/or over-expression of cell cycle promoters. It may be a similar crucial process to the amplification and/or over-expression of *ERBB2* in ERBB2-tumours (Figure 1A). Initial amplifications could happen at 8p11-p12, 8q21-q24, or 20q13 given their high frequencies in luminal B tumours [15,16,24]. This phenomenon would give an acute proliferation advantage to ER-negative CSC, which then undergoes differentiation (luminal features and ER expression). In the tumour development, those cancer clones with higher proliferation activity survive developing new amplifications (Figure 1B), since tumours with enough genomic instability to have an amplification may have an increased probability to arise multiple amplifications [36]. The presence of amplifications in luminal B tumours also correlates with its worse prognosis [16].

What is the difference between those *BRCA1* mutation carriers who develop basal-like and those who develop luminal B carcinomas? We have seen that *BRCA1*-associated luminal B tumours also have LOH (data not shown), so these patients have inactivated both *BRCA1* alleles. It is supposed that these tumours arise from ER-negative SCs [6] or ER-negative PC [7,37], it is plausible that *BRCA1* mutations in ER-negative SCs give rise to basal-like cancers, while in ER-negative PC arise luminal B malignancies. We propose here one other possibility: a different *BRCA1* mutation. Certain BRCA1 mutations give rise to aberrant BRCA1 products if they cause an aminoacid change (missense mutations), or to truncated forms if they produce a stop codon that does not trigger the non-sense mediated decay (NMD) system [38-40]. The presence of these aberrant BRCA1 products could a) have a limited function enabling the cell to differentiate or b) on the contrary, act as a dominant negative and lead to a more aggressive phenotype that those mutations causing a complete absence of the protein [41]. In any case, not all mutations have

necessarily the same phenotypic effect. We have observed in our tumour series that 185delAG mutation in *BRCA1*, which produces a truncated mRNA that avoids NMD-system [39], appears preferentially in *BRCA1*-associated cancers displaying the basal-like phenotype (12/13 185delAG mutation carriers displayed basal-like cancers) (data not shown). However, more samples are needed to confirm this hypothesis. However, a high number of *BRCA1* samples (basal-like and non-basal-like) is needed to address these questions.

Carcinogenesis in an ER-positive progenitor stem cell: the luminal A pathway

Luminal A cancers are present mostly in sporadic and BRCAX-associated cancers, while they are described in a minority of *BRCA2*- and *BRCA1*-associated cancers (Table 1). Although Sorlie and colleagues related *BRCA2* to the luminal A subtype in an expression analysis, only two samples were analyzed, thus the result was not conclusive [17]. The Luminal A phenotype is the least aggressive breast cancer molecular subtype [5], and is also related to low genomic instability according to the studies in sporadic and familial breast cancer subtypes (Figure 2).

Dontu and colleagues, in their CSC hypothesis, postulated that carcinogenesis produced in ER-positive PCs gives rise to ER-positive breast cancers (Figure 1A), which express luminal markers, are composed of more differentiated cells and respond to anti-estrogen treatment [6]. SCs are subject to the accumulation of multiple mutations by their long-lived nature. Therefore, the chance to accumulate a second hit in the wild type allele of the *BRCA1/2* genes is expected to be higher in SCs than in PCs. This could explain the association of *BRCA1/2*-cancers with other phenotypes rather than luminal A. When the second hit is produced in an ER-positive PC, a luminal A phenotype would arise in both *BRCA1/2*-mutation carriers (Figure 1A). Other carcinogenetic events have to be produced to let the carcinogenetic PC acquire self-renewal and other CSC features. Given that PCs are not under BRCA1-driven differentiation regulation, these tumours are able to differentiate and acquire the classical luminal features whether or not the cells have any mutation in *BRCA1* gene (Figure 1B).

Interpretation of the genomic aberrations found in the breast cancer subtypes

Differences in genomic aberrations between breast cancer phenotypes could be evidences of a set of genetic pathways in the breast cancer progression. For example, two different genetic pathways in breast cancer progression have been established using genomic, morphological and IHC features: low-grade, where few genomic changes such as -16q are present, and high grade tumours, where a higher genomic instability is described. Noteworthy, most of the grade I breast tumours do not progress to grade III breast tumours [42,43]

Therefore, we wanted to apply the array-CGH knowledge of breast cancer subtypes to our integrative model in order to elucidate the different genetic pathways. Though each of the breast cancer subtype develops a specific genomic aberration pattern (Figure 2), there are common aberrations that could point out common cell origins or proliferation advantages in concrete cell niches:

Universal genomic aberrations in breast cancer

Data from array-CGH analysis in sporadic [16] and familial breast cancer subtypes [24] have shown strong similarities in the genomic aberration patterns in all breast cancer subtypes. If we consider those genomic imbalances that are present in around 50% of all breast tumour subtypes, we find that the most common genomic aberration is the gain at 1q (Figure 2). This alteration has been described as an early common event in breast cancer in previous conventional-CGH studies [14,44]. More recently, it has also been characterized using array-CGH platforms [45,46]. Orsetti and colleagues described several minimal regions of gains at 1q, which contained genes showing significant over-expression correlated with copy number gains. These genes belonged to pathways such as positive regulation of cell proliferation, transcriptional regulation or chromatin remodeling, and cellular trafficking or basic cellular metabolism [45]. The gain at 1q could be an event required for the deregulation of these pathways and, thus, for enabling the breast carcinogenesis (Figure 1A).

Other genomic aberrations, such as the gain at 8q22-qtel and the loss at 11q23, are relatively common to all breast cancer subtypes. Buerger *et al.* described the gain at 8q as an early genomic event in all breast cancer genetic pathways, probably defining more pleomorphic tumours [44]. However, it is not as frequent in the luminal A subtype as in the other tumour subtypes [15,24], perhaps due to its low histological grade. On the other hand, loss at 11q23 is also recurrent in all familial breast cancer subtypes [24], although its frequency is lower than 50% in ERBB2-and luminal A-sporadic breast cancer subtypes (Figure 2). This genomic region is affected frequently by LOH in breast cancer [47,48], where *ATM* is one of the postulated tumour-suppressor genes that could be targeted [49]. It is possible that these closely common aberrations also play a crucial role in the overall breast tumour development.

Genomic aberrations putatively occurring in Cancer Stem Cell ER-negative

Those breast cancer subtypes that derive from ER-negative CSC (basal-like, ERBB2, and luminal B) could share genomic aberrations, which would not be as recurrent in luminal A tumours (derived from ER-positive CSCs). In this line, the loss at 8p is the most common genomic aberration among these three breast cancer subtypes (both sporadic and familial) (Figure 2).

The CSC hypothesis suggests that the deregulation of the self-renewal pathways in normal mammary is one of the sources for the malignancy of SC. Genetic pathways involved in the self-renewal are Notch, Sonic hedgehog, and Wnt signaling pathways [50]. If tumours that derive from ER-negative CSC share genomic aberrations, it would be possible that these altered regions contain crucial genes that regulate the self-renewal process. Interestingly, when we checked the genes located in 8p, we found two genes at 8p11.21 that were described as down-regulated in breast cancer, constitutively promoting the Wnt signaling pathway: *SFRP1* [51-53] and *DKK4* [54]. Although most of these studies reported the promoter hypermethylation as the silencing mechanism, Veeck and colleagues also described a frequent LOH at *SFRP1* [51] whereas Lo *et al.* reported SFRP1 down-regulation by hyper-methylation in cell lines [53], which also presented losses at 8p in a recent array-CGH analysis [55]. Additionally, *SFRP1* links Hedgehog and Wnt signalling pathways [56,57]. Therefore, the genomic loss of the region that contains

these genes could promote the deregulation of hedgehog and Wnt signaling pathways, and thus, the self-renewal process in ER-negative mammary SC to become CSC (Figure 1A). However, SFRP1 over-expression has been described in basal-like tumours [58]. Moreover, the search for a tumour-suppressor gene in 8p has been extensive without conclusive results, so this reduction to *SFRP1* and *DKK4* roles might result very categorical.

Other common genomic aberrations in basal-like, ERBB2, and luminal B breast cancer are losses at 13qcen-q21 in sporadic, and at 22q in familial tumour subtypes (Figure 2). The loss at 13qcen-q21 could drive to the loss of RB function.

Genomic aberrations associated with estrogen positive cell niche

Different studies have pointed out the genomic aberrations associated with ER status in sporadic [59,60] and familial breast cancers [61]. Under the light of the genomic aberration patterns of each molecular breast cancer subtype, we can distinguish a heterogeneity within each ER-status group. Basal-like and ERBB2 breast subtypes, though being all ER-negative tumours, have clearly different genomic aberration patterns; and the same can be applied to ER-positive tumours (luminal A and B subtypes) (Figure 2). Indeed, aberrations classically associated with ER-negative tumours can be specifically attributable to basal-like tumours such as +3q25-qtel, -4p, -4q22-qtel, -5q, etc. This association is explained by an over-representation of basal-like tumours in the studied ER-negative tumour collections. On the other hand, genomic aberrations in luminal B tumours differed clearly from luminal A malignancies, probably due to the different carcinogenesis and tumour development. However, there are common genomic aberrations present in these two breast cancer subtypes: +16p, -16q, and amplification at 11q13 (*CCND1* locus) which are not as frequent in the other tumour subtypes. We think that these genomic aberrations might give a proliferation advantage in an ER-positive cell niche.

The loss at 16q seems to be an initial event in lobular and low-grade carcinomas, while it is considered a secondary event in high-grade carcinomas probably due to a high overall genomic instability. This chromosomal aberration is in fact one of the few genomic changes in low-grade tumours [42]. Recently, an array-CGH analysis of the rearrangements at 16q discriminated the aberrations occurred in low- and high-grade tumours [62]. *CDH1* is the putative tumour suppressor gene at 16q22.1, which has been related to lobular and low–grade tumours, but other tumour suppressor genes are supposed to be targeted in high-grade carcinomas (reviewed in [63]). As mentioned above, there is a controversy about the origin of low-and high-grade breast invasive carcinomas reviewed in different studies. Two different genetic pathways are supposed to give rise to grade I and grade II-III tumours. [42-44]. In this line, as luminal A tumours are preferentially low-grade carcinomas and luminal B are grade II or III [24,58,64], loss at 16q could be produced in these two tumour subtypes by different mechanisms. In luminal A tumours, it would be an early genomic event whereas in luminal B cancers, it would be produced by the genomic instability. However, given the high aberration frequency in both tumour subtypes, the loss at 16q maybe crucial in the carcinogenesis and tumour development under an ER-positive cell niche.

The amplification at 11q13 (*CCND1* locus) is another common aberration present in luminal A and B cancer phenotypes. A strong correlation between *CCND1* amplification and over-expression has been reported. CCND1 over-expression has been associated with ER expression and it is rarely present in basal-like carcinomas [65,66]. Our analysis in familial breast cancer subtypes also showed that CCND1 over-expression was associated with luminal tumours, and inversely correlated with basal-like cancers [24]. These findings suggest that CCND1 does not play an important role in the basal-like tumour development, whereas it seems important in the luminal tumour genetic pathways.

SUMMARY

We propose an integrative model of breast carcinogenesis and tumour development, which includes concepts such as the *BRCA1/2* genes and shared genomic aberrations, and supports a comprehensive model of CSC hypothesis and clonal selection [67]. As represented in Figure 1, ER-negative SCs/PCs could be targeted for carcinogenesis by several events: a) *BRCA1* silencing that produces differentiation arrest and thus, a basal-like phenotype, b) *ERBB2* amplification restricted to non-*BRCA1/2*-affected patients, and c) crucial proliferation advantages probably due to other amplifications different than ERBB2-tumours in luminal B cancers. On the other hand, carcinogenesis in ER-positive PCs generate luminal A tumours. To further support this hypothesis, array-CGH analyses describe common genomic aberrations required for the breast tumour development (+1q, -11q23, etc.), for the ER-negative CSC population (-8p), and for tumour cells confined to an ER-positive cell niche (-16p).

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Table 1. Proportion of breast cancer subtype in sporadic, *BRCA1*-, *BRCA2*-, and BRCAX-associated cancers according to previous studies.

Breast cancer class	Breast cancer subtype (%)					Classification tool	Reference
	Basal-like	ERBB2	Luminal B	Luminal A	Other†		
Sporadic	16.5	12.9	17.6	37.7	15.3	Intrinsic gene list	Sorlie *et al*, 2001
	15.6	18.9	13.9	41.8	9.8	Intrinsic gene list	Carey *et al*, 2006
	26.7	9.2	19.0	28.6	16.5	Intrinsic gene list	Hu *et al*, 2006
	25.2	9.4	20.8	46.2	-	IHC††	Callagy *et al,* 2003
	16.0	16.0	20.0	36.0	12.0	IHC††	Honrado *et al,* 2007
BRCA1	88.9	-	-	-	11.1	Intrinsic gene list	Sorlie *et al,* 2003
	61.1	-	22.2	5.6	11.1	IHC††	Melchor *et al,* in press
BRCA2	-	-	-	100†††	-	Intrinsic gene list	Sorlie *et al,* 2003
	18.8	-	37.5	12.5	31.2	IHC††	Melchor *et al,* in press
BRCAX	14.0	18.0	14.0	36.0	18.0	IHC††	Honrado *et al,* 2007
	7.1	14.3	25.0	46.5	7.1	IHC††	Melchor *et al,* in press

†, "other" breast cancer subtype column compiles "normal breast-like", "interferon-like", and other "unclassified" groups described in these analyses.
††, Callagy et al. used a panel of 12 antibodies [64], while we used 25 antibodies and histological grade to profile breast tumour classes [22,24] Another IHC analysis profiling breast cancer classes was performed by Oldenburg *et al*. The authors also discriminated BRCA-mutation carriers, but they only used basal and luminal cytokeratins to discriminate among tumour groups, so a correlation with Sorlie's subtype is not easily established [68].
†††, only two BRCA2 cases were studied.

FIGURE LEGENDS

Figure 1. Integrative model of carcinogenesis and tumour development in mammary stem cells. 1A, different carcinogenetic events and breast cancer classes are represented, yellow boxes of breast cancer classes represent the most frequent pathway that the breast cancer class develops. Crossed boxes of *BRCA1/2*-mutation carriers in ERBB2 phenotype pathway represent their inability to undergo this genomic pathway. Universal genomic changes are shown on each pathway, and also those "initial genomic events" in ER-negative CSC and ER-positive PC. 1B, set of phenomena that occur during the tumour development such as differentiation processes, acquisition of genomic aberrations, and other tumourogenic events. Genomic instability is also represented as arrows in the bottom, which thickness represents the level of instability.

Figure 2. Genomic aberration patterns of sporadic and familial breast cancer subtypes adapted from two previous studies [16,24]. (●) means universal genomic aberrations in all breast cancer subtypes, (**Φ**) underlines regions commonly affected in subtypes putatively originated from ER-negative CSC, and (*) represents genomic aberrations recurrently present in ER-positive tumours.

Figure 1

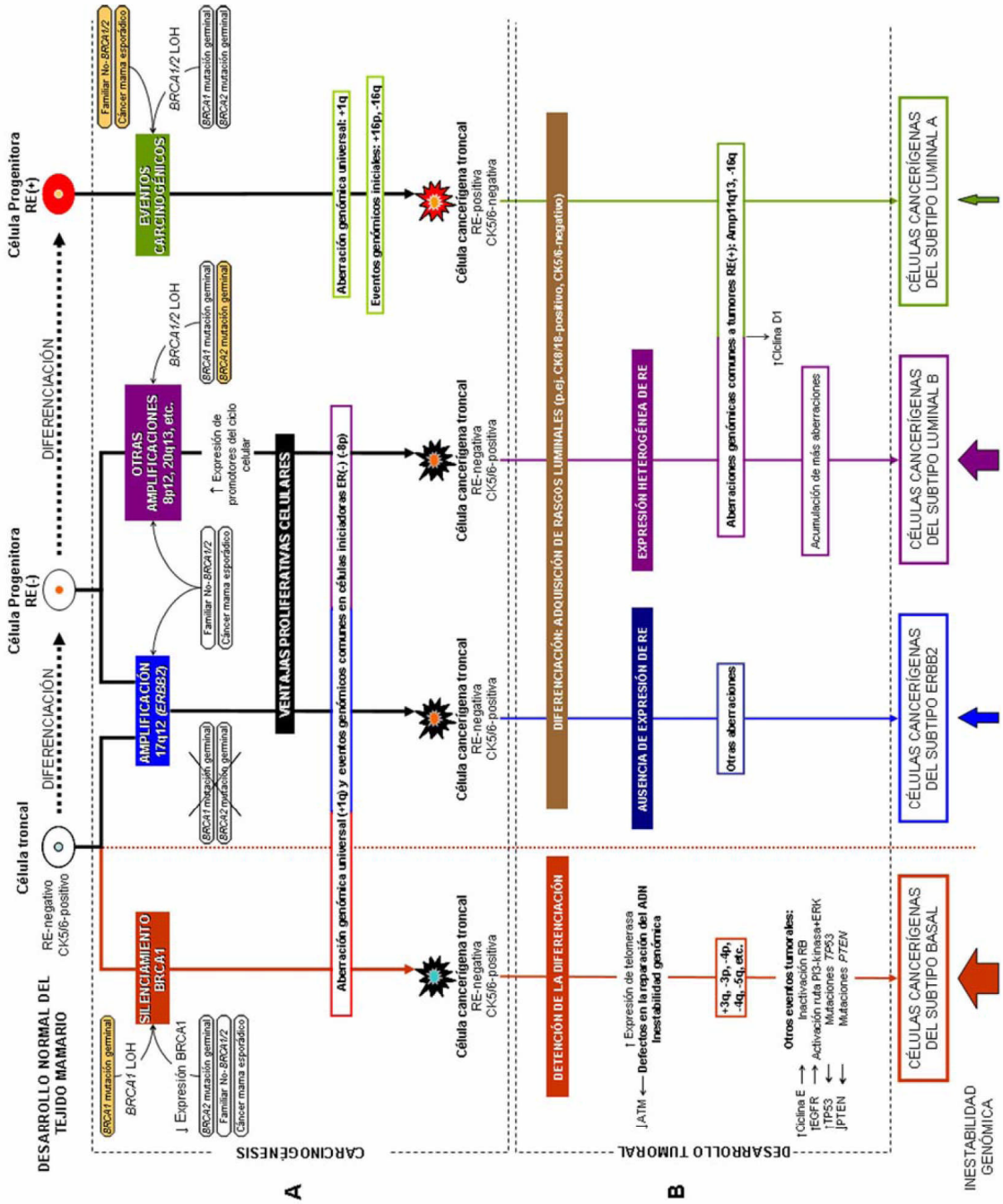
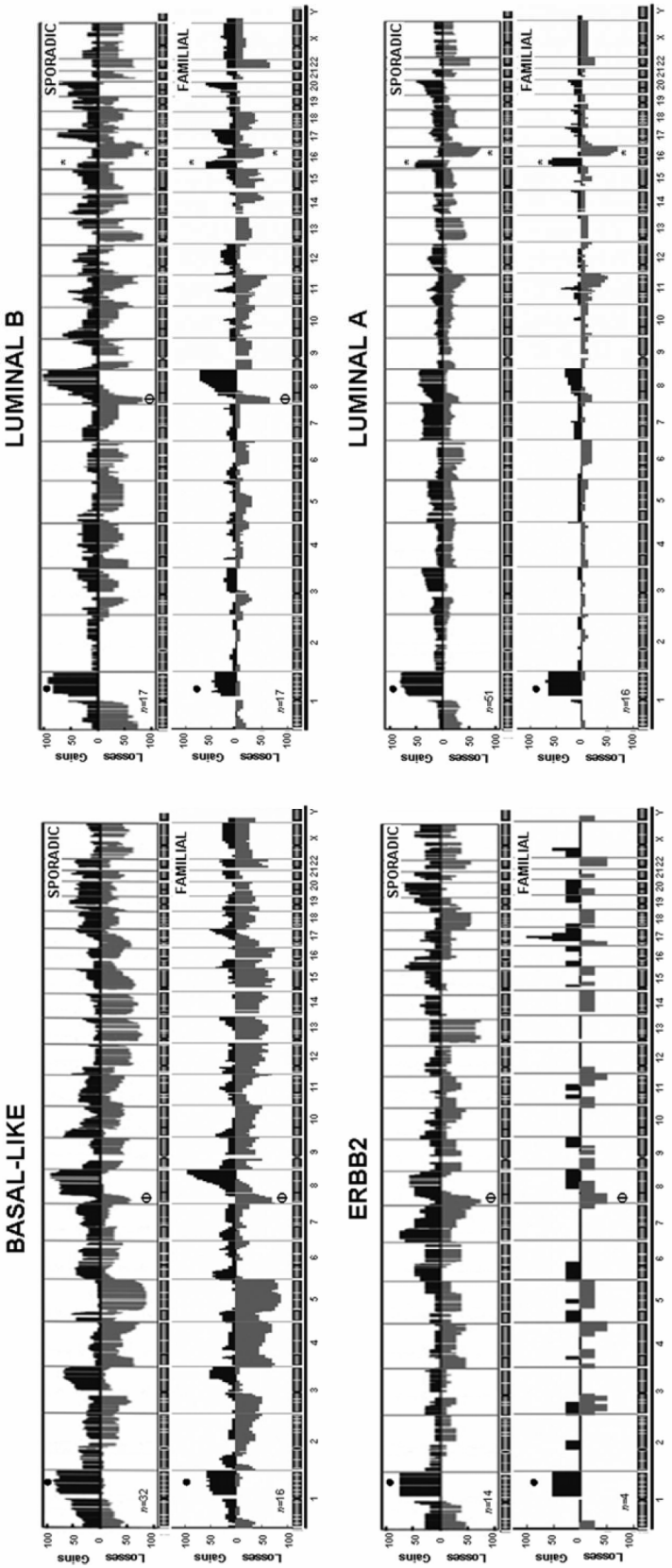

DESARROLLO NORMAL DEL TEJIDO MAMARIO
Célula troncal
RE-negativo CK5/6-positivo
DIFERENCIACIÓN
Célula Progenitora RE(-)
DIFERENCIACIÓN
Célula Progenitora RE(+)
SILENCIAMIENTO BRCA1
BRCA1 LOH
↓ Expresión BRCA1
BRCA1 mutación germinal
BRCA2 mutación germinal
Familiar No-BRCA1/2
Cáncer mama esporádico
AMPLIFICACIÓN 17q12 (ERBB2)
OTRAS AMPLIFICACIONES 8p12, 20q13, etc.
↑ Expresión de promotores del ciclo celular
EVENTOS CARCINOGÉNICOS
Familiar No-BRCA1/2
Cáncer mama esporádico
BRCA1/2 LOH
BRCA1 mutación germinal
BRCA2 mutación germinal
VENTAJAS PROLIFERATIVAS CELULARES
Aberración genómica universal (+1q) y eventos genómicos comunes en células iniciadoras ER(-) (-8p)
Aberración genómica universal: +1q
Eventos genómicos iniciales: +16p, -16q
Célula cancerígena troncal RE-negativa CK5/6-positiva
Célula cancerígena troncal RE-positiva CK5/6-negativa
DIFERENCIACIÓN: ADQUISICIÓN DE RASGOS LUMINALES (p.ej. CK8/18-positivo, CK5/6-negativo)
EXPRESIÓN HETEROGÉNEA DE RE
AUSENCIA DE EXPRESIÓN DE RE
Otras aberraciones
Aberraciones genómicas comunes a tumores RE(+): Amp11q13, -16q
↑Ciclina D1
Acumulación de más aberraciones
DETENCIÓN DE LA DIFERENCIACIÓN
↑ Expresión de telomerasa
↓ATM ← Defectos en la reparación del ADN
Inestabilidad genómica
+3q, -3p, -4p, -4q, -5q, etc.
Otros eventos tumorales:
↑Ciclina E → Inactivación RB
↑EGFR → Activación ruta PI3-kinasa+ERK
↑TP53 ↔ Mutaciones TP53
↓PTEN ↔ Mutaciones PTEN
CÉLULAS CANCERÍGENAS DEL SUBTIPO BASAL
CÉLULAS CANCERÍGENAS DEL SUBTIPO ERBB2
CÉLULAS CANCERÍGENAS DEL SUBTIPO LUMINAL B
CÉLULAS CANCERÍGENAS DEL SUBTIPO LUMINAL A
INESTABILIDAD GENÓMICA
CARCINOGÉNESIS
DESARROLLO TUMORAL
A
B

Figure 2


LUMINAL B
SPORADIC
FAMILIAL
LUMINAL A
SPORADIC
FAMILIAL
BASAL-LIKE
SPORADIC
FAMILIAL
ERBB2
SPORADIC
FAMILIAL
Losses
Gains

ANEXO III: PUBLICACIONES RELACIONADAS CON LA TESIS

A continuación se exponen la referencia y un breve resumen de aquellos trabajos en los que el doctorando ha estado involucrado durante su periodo predoctoral y que se relacionan con el tema de la tesis.

Clin Cancer Res. 2005 Feb 1;11(3):1146-53.

A predictor based on the somatic genomic changes of the BRCA1/BRCA2 breast cancer tumors identifies the non-BRCA1/BRCA2 tumors with BRCA1 promoter hypermethylation.

Alvarez S, Diaz-Uriarte R, Osorio A, Barroso A, Melchor L, Paz MF, Honrado E, Rodríguez R, Urioste M, Valle L, Díez O, Cigudosa JC, Dopazo J, Esteller M, Benitez J.

The genetic changes underlying in the development and progression of familial breast cancer are poorly understood. To identify a somatic genetic signature of tumor progression for each familial group, BRCA1, BRCA2, and non-BRCA1/BRCA2 (BRCAX) tumors, by high-resolution comparative genomic hybridization, we have analyzed 77 tumors previously characterized for BRCA1 and BRCA2 germline mutations. Based on a combination of the somatic genetic changes observed at the six most different chromosomal regions and the status of the estrogen receptor, we developed using random forests a molecular classifier, which assigns to a given tumor a probability to belong either to the BRCA1 or to the BRCA2 class. Because 76.5% (26 of 34) of the BRCAX cases were classified with our predictor to the BRCA1 class with a probability of >50%, we analyzed the BRCA1 promoter region for aberrant methylation in all the BRCAX cases. We found that 15 of the 34 BRCAX analyzed tumors had hypermethylation of the BRCA1 gene. When we considered the predictor, we observed that all the cases with this epigenetic event were assigned to the BRCA1 class with a probability of >50%. Interestingly, 84.6% of the cases (11 of 13) assigned to the BRCA1 class with a probability >80% had an aberrant methylation of the BRCA1 promoter. This fact suggests that somatic BRCA1 inactivation could modify the profile of tumor progression in most of the BRCAX cases.

Hum Mutat. 2006 Mar;27(3):242-8.

A haplotype containing the p53 polymorphisms Ins16bp and Arg72Pro modifies cancer risk in BRCA2 mutation carriers.

Osorio A, Martínez-Delgado B, Pollán M, Cuadros M, Urioste M, Torrenteras C, Melchor L, Díez O, De La Hoya M, Velasco E, González-Sarmiento R, Caldés T, Alonso C, Benítez J.

Germline mutations in the BRCA1 and BRCA2 genes confer a high lifetime risk of developing breast and other cancers; however, remarkable differences exist regarding disease manifestation in mutation carriers. It has been suggested that other genetic and/or environmental factors modify not only the appearance but also the age of onset and type of tumor in BRCA1/2-associated cases. The aim of the present study was to investigate the role of two p53 polymorphisms (c.97-147ins16bp and c.215c>g, p.Arg72Pro) as potential modifiers. For this purpose we investigated the possible association between the two polymorphisms and disease status in 447 BRCA1/2 mutation carriers belonging to 170 Spanish breast and/or ovarian cancer families. Genotype and haplotype analyses revealed that the presence of a specific haplotype carrying the allele without the 16-bp insertion and the variant allele for the Arg72Pro (No Ins-72Pro haplotype) was associated with an earlier age of onset in BRCA2 mutation carriers. We found an increased risk of developing a first primary tumor (breast or ovarian) before 35 years of age for individuals who carried at least one No Ins-72Pro haplotype (OR: 2.69; 95% CI: 1.15-6.29; P=0.022). We confirmed these data by a functional study in which we compared different p53 genotypes in relation to their apoptotic response after cell treatment with a cytotoxic drug (AraC). Our results revealed a decrease in p53 apoptotic rate associated with the No Ins-72Pro haplotype.

Mod Pathol. 2007 Dec;20(12):1298-306

Immunohistochemical classification of non-BRCA1/2 tumors identifies different groups that demonstrate the heterogeneity of BRCAX families.

Honrado E, Osorio A, Milne RL, Paz MF, Melchor L, Cascón A, Urioste M, Cazorla A, Díez O, Lerma E, Esteller M, Palacios J, Benítez J.

Around 25% of hereditary breast and ovarian cancer families have mutations in the BRCA1 and BRCA2 genes. The search for other genes has until now failed, probably because there is not one single BRCAX gene, but rather various genes that may each be responsible for a small number of breast cancer families and/or may interact according to a polygenic model. We have studied 50 tumors from probands belonging to non-BRCA1/2 breast cancer families (BRCAX), using 25 immunohistochemical markers. The objective was to classify these tumors and confirm that they are heterogeneous. Unsupervised cluster analysis showed the existence of the following two main groups of tumors: high-grade and estrogen receptor (ER)-negative tumors (50%), and low-grade and ER-positive tumors (50%). In addition we identified five subgroups, three among the high-grade and two among the low-grade groups; one overexpressing HER-2 (18%); one with a basal-like phenotype (14%); one with a normal breast-like phenotype (18%); a luminal A subgroup (36%), and a luminal B subgroup (14%). Hypermethylation of the BRCA1 gene was observed in 42% of the cases, spread across all five subgroups, but only 37% of those had loss of heterozygosity as well. These latter cases were all clustered in the high-grade group and the majority of them in the basal-like subgroup. Our results show that familial non-BRCA1/2 tumors are heterogeneous and suggest a polygenic model for explaining the majority of BRCAX families. In addition we have defined a subset of them that have somatic inactivation of the BRCA1 gene.

Manuscript in preparation

Detection of a pattern of artifactual copy number variations that can induce to overestimate changes on genome profiling analysis

Blesa D, Suela J, Melchor L, Álvarez-de-Andres S, Largo C, Ferreira BI, Cifuentes F, Benitez J, Cigudosa JC.

Copy number changes affecting certain regions of the genome are the basis of many congenital diseases and are highly prevalent in tumor samples of all types. In this report we show a specific genomic and chromosomic pattern of artifactual copy number variations that we have detected and characterized. This artifact has been detected using standard array-CGH technology for genome profiling studies. We have found an association between the artifactual genomic pattern and regional gene density, which correlates with the GC content of genomic DNA. Regional GC content could be the genome-scale driving characteristic that causes the observed genomic/chromosomic pattern but still remains elusive a sample factor that promotes the appearance of the artifact. Following current standard procedures, 10% of DNA samples extracted from fresh tissues and about 85% of DNA extracts from formalin-fixed paraffin embedded samples produced the artifact. We think that the artifact is therefore going unnoticed into the reported literature. Until a cause is established the only way of evaluating the presence/absence of the artifact is the inspection of the aCGH genomic profile and recognition of the characteristic pattern described in this report. Any copy number variation detected by arrayCGH analysis should be compared with the artifactual pattern presented in this report. Our results provide the basis to develop software tools able to detect and normalize artifactual data.

ANEXO IV: OTRAS PUBLICACIONES DEL DOCTORANDO

Genes Chromosomes Cancer 2005 Mar;42(3):287-98.

Analysis of myelodysplastic syndromes with complex karyotypes by high-resolution comparative genomic hybridization and subtelomeric CGH array.

Martínez-Ramírez A, Urioste M, Melchor L, Blesa D, Valle L, de Andrés SA, Kok K, Calasanz MJ, Cigudosa JC, Benítez J.

Molecular cytogenetic techniques enabled us to clarify numerical and structural alterations previously detected by conventional cytogenetic techniques in 37 patients who had myelodysplastic syndromes with complex karyotypes. Using high-resolution comparative genomic hybridization (HR-CGH), we found the most recurrent alterations to be deletion of 5q (70%), 18q (35%), 7q (32%), 11q (30%), and 20q (24%), gain of 11q (35%) and 8q (24%), and trisomy of chromosome 8 (19%). Furthermore, in 35% of the patients, 20 amplifications were identified. These amplifications were shown by FISH to involve some genes previously described as amplified in hematological malignancies, such as ERBB2, MLL, and RUNX1. In addition, two other genes, BCL6 and BCL2, which are classically related to apoptosis and non-Hodgkin lymphoma, were shown for the first time to be involved in amplification. Genomic alterations involving different subtelomeric regions with losses in 4p16, 5p15.3, 6q27, 18p11.3, and 18q23 and gains in 1p36.3 and 19p13.3 were detected by HR-CGH. Array CGH analysis of the subtelomeric regions in some samples was able to confirm a number of these alterations and found some additional alterations not detected by conventional CGH.

Eur J Hum Genet. 2005 May;13(5):570-8.

About the origin and development of hereditary conventional renal cell carcinoma in a four-generation t(3;8)(p14.1;q24.23) family.

Valle L, Cascón A, Melchor L, Otero I, Rodríguez-Perales S, Sánchez L, Cruz Cigudosa J, Robledo M, Weber B, Urioste M, Benítez J.

Conventional renal cell carcinoma (CRCC) may appear in families with germline translocations involving chromosome 3, although a recurrent responsible gene has not been found. We recently described a family with CRCC and a constitutional t(3;8)(p14.1;q24.23), and we demonstrated that no genes were disrupted by the translocation breakpoints. In order to investigate the genetic origin and features of the CRCC tumors that occurred in this family, we have extended the pedigree up to four generations, and analyzed peripheral blood samples from 36 members, CRCC tumors, normal renal tissues, and a gastric tumor. (1) By means of comparative genomic hybridization (CGH), we have detected loss of the derivative chromosome carrying 3p in all CRCC but not in the corresponding normal renal tissue. In addition, by means of the fluorescence in situ hybridization technique, we have observed that not all tumoral cells lose the der(3p), which suggests that, previous to this loss, another hit should occur to initiate the transformation of normal into tumoral cells. (2) All known mechanisms of inactivation of the candidate von Hippel-Lindau (VHL) gene have been studied in the tumors, detecting alterations in 65% of them. This confirms that inactivation of the VHL gene is not always required to develop CRCC, and that (an)other suppressor gene(s) on 3p could be involved. (3) We discard FHIT as an alternative pathway to VHL. We have not found new candidate regions along 3p by using a 1-Mb resolution array-based CGH. (4) The tumorigenesis mechanism of a second gastric tumor developed in the probandus is different from that of CRCC.

A Predictor Based on the Somatic Genomic Changes of the BRCA1/BRCA2 Breast Cancer Tumors Identifies the Non-BRCA1/BRCA2 Tumors with BRCA1 Promoter Hypermethylation

Sara Alvarez,[1] Ramon Diaz-Uriarte,[3] Ana Osorio,[1] Alicia Barroso,[1] Lorenzo Melchor,[1] Maria Fe Paz,[2] Emiliano Honrado,[1] Raquel Rodríguez,[1] Miguel Urioste,[1] Laura Valle,[1] Orland Díez,[5] Juan Cruz Cigudosa,[4] Joaquin Dopazo,[3] Manel Esteller,[2] and Javier Benitez[1]

[1]Human Genetics Department and [2]Cancer Epigenetics Group, Molecular Pathology Programme, and [3]Bioinformatics Unit and [4]Cytogenetics Unit, Biotechnology Programme, Spanish National Cancer Centre, Madrid, Spain; and [5]Department of Genetics, H. Santa Creu i Sant Pau, Barcelona, Spain

ABSTRACT

The genetic changes underlying in the development and progression of familial breast cancer are poorly understood. To identify a somatic genetic signature of tumor progression for each familial group, BRCA1, BRCA2, and non-BRCA1/BRCA2 (BRCAX) tumors, by high-resolution comparative genomic hybridization, we have analyzed 77 tumors previously characterized for *BRCA1* and *BRCA2* germ line mutations. Based on a combination of the somatic genetic changes observed at the six most different chromosomal regions and the status of the estrogen receptor, we developed using random forests a molecular classifier, which assigns to a given tumor a probability to belong either to the BRCA1 or to the BRCA2 class. Because 76.5% (26 of 34) of the BRCAX cases were classified with our predictor to the BRCA1 class with a probability of >50%, we analyzed the BRCA1 promoter region for aberrant methylation in all the BRCAX cases. We found that 15 of the 34 BRCAX analyzed tumors had hypermethylation of the *BRCA1* gene. When we considered the predictor, we observed that all the cases with this epigenetic event were assigned to the BRCA1 class with a probability of >50%. Interestingly, 84.6% of the cases (11 of 13) assigned to the BRCA1 class with a probability >80% had an aberrant methylation of the *BRCA1* promoter. This fact suggests that somatic *BRCA1* inactivation could modify the profile of tumor progression in most of the BRCAX cases.

INTRODUCTION

Inheritance of a mutant *BRCA1* (OMIM#113705) or *BRCA2* (OMIM#600185) gene confers a lifetime 50% to 85% risk of breast cancer and a 15% to 45% risk of ovarian cancer (1, 2). At present, high-risk families are usually selected for genomic screening for *BRCA1* and *BRCA2* germ line mutations based on a set of criteria, including family history, age of onset, and occurrence of ovarian carcinomas in the family. However, germ line mutations are not identified in 70% of the families fulfilling these criteria (3, 4). Genetic linkage analysis of these non-BRCA1/BRCA2 families (termed BRCAX families) has been done, and several chromosomal regions were identified, including 8p12-p22 (5) and 13q21 (6), potentially harboring breast cancer susceptibility genes. However, these loci have been subsequently excluded as major predisposing loci on a global perspective (7, 8), emphasizing the genetic heterogeneity and population-specific effects within BRCAX kindred (4).

Breast carcinomas from patients with a germ line mutation in *BRCA1* or *BRCA2* show some typical pathologic characteristics. BRCA1 tumors are generally high-grade, steroid receptors and HER-2 negative and p53 protein positive compared with controls. In contrast, BRCA2 tumors do not show significant differences in the expression of these proteins compared with control tumors (9). In addition, we have identified specific differences between BRCAX and BRCA1 tumors. BRCAX cases generally show lower-grade, positive steroid receptors and BCL2, negative p53, low proliferation rate, and absence of P-cadherin expression. However, only lower grade and lower proliferation rate differentiate the BRCAX tumors from the BRCA2 and control group (10).

In addition to the pathologic differences, breast tumors in carriers of mutant *BRCA1* or *BRCA2* gene are characterized by a large number of chromosomal changes, some of which differ from sporadic tumors depending on their genotype (11, 12). Losses of 5q, 4q, 4p, 2q, and 12q in BRCA1 tumors and losses of 13q and 6q and gains of 17q22-24 and 20q13 in BRCA2 tumors were significantly more common than in controls (11). Recently, some of these changes, such as loss of 5q and 12q, have been confirmed (12). Then, based on the genomic changes observed using comparative genomic

Received 6/14/04; revised 11/2/04; accepted 11/11/04.
Grant support: Ayto de Madrid-CNIO, Ramon y Cajal Programme from the Spanish MCyT (R. Diaz-Uriarte), Fundación La Caixa grant BIO2001-0068, MCyT grants SAF03-02497 and SAF01-0075, and Red Temática de Investigación Corporativa de Centros de Cancer grant C0-010.
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hybridization (CGH), the BRCA1 profile has been used to build a classifier that distinguished between sporadic and BRCA1 tumors (12).

In the present work, we have used a more refined genome-wide approach as high-resolution CGH (HR-CGH) that more precisely identify the somatic genetic changes associated to BRCA1, BRCA2, and non-BRCA1/BRCA2 germ line mutation carriers. Based on the distinctive profiles of tumor progression among BRCA1/BRCA2 mutation carriers, we have build a classifier that allow for the first time to differentiate BRCA1 and BRCA2 tumors and to define the differences and similarities of the BRCAX tumors compared with the BRCA1/BRCA2 classes.

MATERIALS AND METHODS

Patients and Tumor Samples. Patients were referred to the Spanish National Cancer Center from the Fundación Jimenez-Díaz in Madrid or from the Hospital San Pau in Barcelona for genetic studies. Seventy-seven breast cancer tumors from patients with BRCA1 germ line mutation (24 cases) and BRCA2 (19 cases) or without BRCA1/BRCA2 mutations (34 cases) were selected from families with at least three women affected with breast cancer, one of them <50 years old, or with ovarian cancer or with male breast cancer (13–15). The characteristics of these patients are summarized in the supplemental research data (Table 1). Mutation analysis of *BRCA1* and *BRCA2* genes of all the cases has been done as described previously (3, 15).

CGH Analysis. Genomic DNA was isolated from 4 × 10 μm sections of 77 paraffin-embedded tumors using the commercially available DNase mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's recommendations. CGH was done as described previously with minor modifications (16) Briefly, tumor DNAs were labeled with spectrum green dUTP using the nick translation labeling kit (Vysis). Commercially available normal male DNA labeled with Texas Red dUTP was used as control reference (Vysis). Images of the hybridized normal male metaphases were evaluated with a digital image analysis system based on an Olympus AX60 epifluorescence microscope and a cooled CCD camera (Photometrics, Inc., Tucson, AZ) interfaced to a Cytovision Image Analysis System (Applied Imaging, Newcastle, United Kingdom). Three-color images were acquired from 10 to 15 metaphases per specimen. Calculation of green to red fluorescence ratios along the length of the chromosomes was done using the Cytovision system version 2.7 HR-CGH analysis software. The CGH profiles were compared with a dynamic standard reference interval based on an average of normal cases as described by Kirchhoff et al. (17). The mean ratio profile of each case with 95% confidence intervals was compared with the average ratio profile of the normal cases with similar 95% confidence intervals. Positive findings were considered those where the 95% confidence intervals of the patient profile and normal averaged profile did not overlap.

Hybridizations of normal breast tissue paraffin-embedded DNA versus reference female DNA were used as negative controls previous to the samples analysis. Because the tumor and reference DNA were not sex matched, the X and Y chromosomes were omitted from the analysis.

Statistical Analysis of CGH Data. A nonparametric Mann-Whitney *U* test was used to identify differences in the number of chromosomal gains and losses among the three patient groups. Differences in the frequency of involvement of individual chromosomal regions among the three familial breast cancer classes and among the BRCA1 and BRCA2 group with recurrent versus nonrecurrent mutations were tested with Fisher's exact test. The indicated *P*s were not corrected for multiple testing and were calculated using the Stat POMELO tool available at http://pomelo.bioinfo.cnio.es (18).

Building a Predictor Data Preprocessing. Because a high number of variables better defined the tumor progression profile of each group, we selected 63 G-banded cytogenetic regions to refine the detected HR-CGH profile (Fig. 1). We have chosen as the most common minimal regions of involvement 50 regions including imbalances in at least 30% of the 28 BRCA1/BRCA2 cases used to build the predictor and with at least 3 cases defining the cytogenetic thresholds. To include the rest of the genome not fitting the previously defined criteria, we grouped the unselected areas on 13 chromosomal regions.

We selected the 63 previously defined chromosomal regions and some pathologic variables, such tumor grade and steroid receptor status, previously reported to behave differentially as potential predictors. A discrete value was assigned to each region for each tumor. A particular region had three possible discrete values, gain, loss, or no change. For the steroid receptor status, we recode them as positive (cases with >9% of positivity) or negative (10, 19). For grade, we impute the missing values to the most frequent variable value.

Analyses. We have used random forests to obtain a simple and good predictor of BRCA1 and BRCA2. Other approaches attempted without success are detailed in supporting

Table 1 Chromosomal regions with significant differences between BRCA1 and BRCA2 cases used to build the predictor (Fisher's exact test, not corrected for multiple testing, $P < 0.05$)

	Percentage of affected tumors			*P*		
Regions	BRCA1 ($n = 15$)	BRCA2 ($n = 13$)	BRCAX ($n = 34$)	BRCA1 vs. BRCA2	BRCA1 vs. BRCAX	BRCA2 vs. BRCAX
8p11-12 Gain	13.3	69.2	29.41	0.005	NS	0.020
12q11-21 Gain	20	76.9	41.18	0.006	NS	0.048
2p11-21 Loss	0	30.7	8.8	0.042	NS	NS
15q22-26 Loss	53.3	7.7	55.9	0.014	NS	0.003
18q Loss	60	15.4	55.9	0.020	NS	0.020

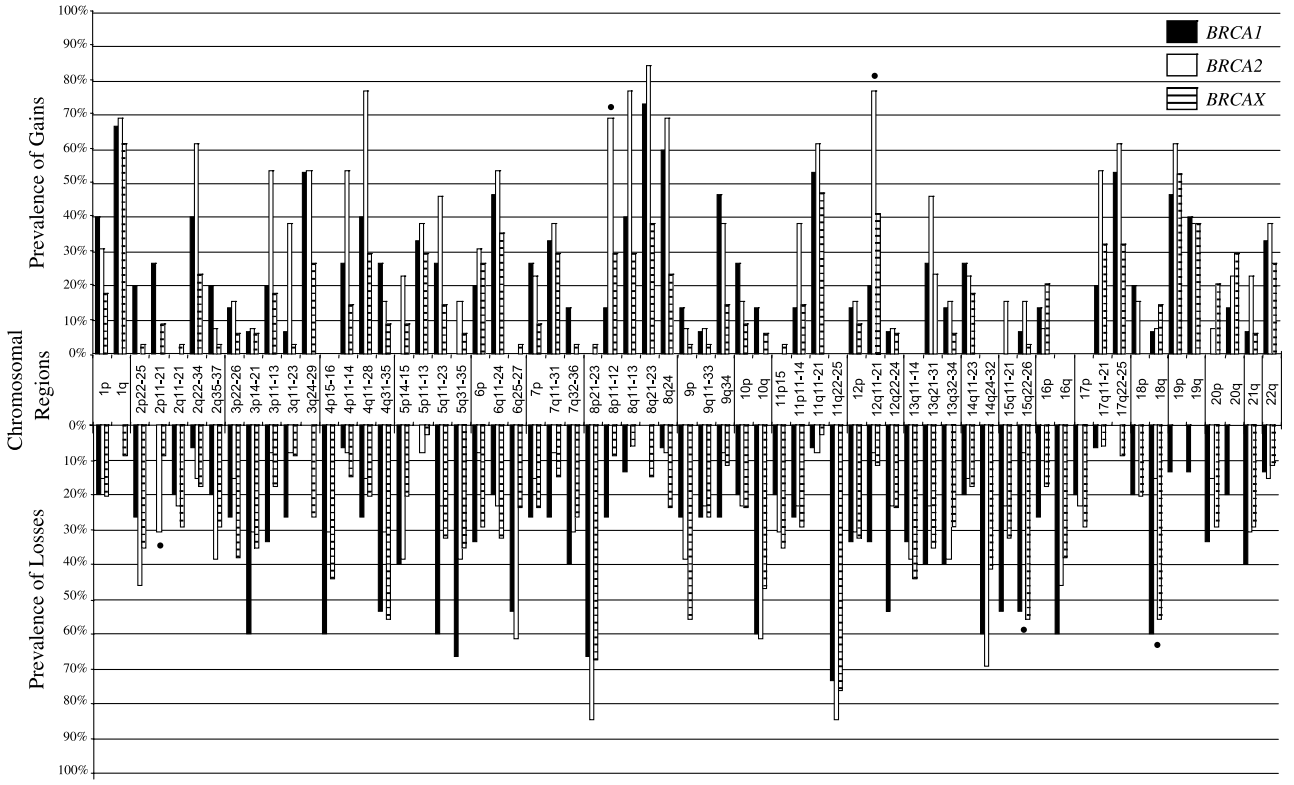

Fig. 1 Distribution of the copy number changes detected by CGH in 62 familial breast tumors analyzed on the 63 selected chromosomal regions. Above and below the X axis, gains and losses, respectively. •, chromosomal regions with a significantly different frequency of involvement (Fisher's exact test, not corrected for multiple sampling, $P < 0.05$) between BRCA1 and BRCA2.

information. Random forests are essentially an ensemble of classification trees that can be used directly with several variables much larger than the number of samples and that often show very good predictive performance (20, 21).

Random forests return a prediction as the unweighed majority of predictions from a very large (e.g., 5,000) collection of classification trees (22); the important features about these trees are that each one is grown using a bootstrap sample of the data set and that, at each node, only a random subset of the original variables is examined. As part of a random forests run, we obtained measures of the importance of the variables. A large importance measure indicates that random permutation of that variable causes samples to be misclassified more often (hence, that variable is important). Although random forests provide a very good classification and return a ranking of the importance of variables, a predictor built from 66 predictor variables is cumbersome to use and very difficult to understand. Moreover, elimination of variables that do not contribute to the classification could actually result in an improved predictive performance. Our approach to variable selection is very similar to that of Svetnik et al.[6]; essentially, we iteratively eliminate the variable with a lower importance measure until the out-of-bag estimate of prediction error becomes larger than the out-of-bag estimate of prediction error of either the previous model or the original model. However, these out-of-bag estimates are biased down because of the variable selection process (23–25). We have thus estimated the prediction error of our simplification procedure using a leave-one-out cross-validation of the complete variable selection process; in other words, excluding each observation in turn, we run the complete variable selection procedure and then predict the left-out observation. This, therefore, yields a honest, almost unbiased estimate of prediction error, because the error rate is computed from observations that were never used for the simplification and building of the forest that is predicting an observation. For the analyses, we have used the random forests package for R (by A. Liaw and M. Wiener) that uses the Fortran random forests code by L. Breiman and A. Cutler. The R code for the variable selection is available from authors on request. The predictor will be available at http://bioinfo.cnio.es/data.

Methylation. A methylation-specific PCR assay was used to distinguish unmethylated alleles from methylated alleles of *BRCA1* based on sequence changes produced by treating DNA with bisulfite, which converts unmethylated (but not methylated) cytosines to uracil followed by a PCR assay involving primers designed for either methylated or unmethylated DNA (26). The methylated product is 75 bp long and the unmethylated one is 86 bp. DNA from normal lymphocytes was used as a negative control, and *in vitro* methylated DNA was used as a positive control (Fig. 3E).

[6] V. Svetnik et al. Variable selection in random forest with application to quantitative structure-activity relationship, in preparation 2005.

RESULTS

Overall Genetic Changes in the Three Groups of Familial Breast Cancer Tumors. The HR-CGH profiles of 15 BRCA1 (cases 1-15; supplemental research data; Table 1), 13 BRCA2 (cases 25-37; supplemental research data; Table 1), and 34 BRCAX breast carcinomas were analyzed at the 63 selected chromosomal regions (Fig. 1; see Building a Predictor). The selection of these regions was possible due to the improved sensitivity and specificity of the HR-CGH software (27, 28). For example, we could effectively discriminate changes affecting different regions within the short arm of chromosome 8 (Fig. 2). In fact, loss of 8p21-23 region was observed in >60% of the cases in the three groups, whereas gain at 8p11-12 band was present in 69% of the BRCA2 cases and in <30% of the BRCA1 and BRCAX tumors (Fig. 1).

The mean ± SE number of genetic changes was similar among the three groups, 32.3 ± 3, 31.6 ± 3.2, and 26.5 ± 1.7 for BRCA1, BRCA2, and BRCAX, respectively. However, a higher number of DNA losses in the BRCA1 (19 ± 1.9) and BRCAX (16.53 ± 1.43) cases compared with the BRCA2 group (13.8 ± 2.7) and a lower number of gains in the BRCA1 (13.3 ± 1.9) and BRCAX tumors (10 ± 1.8) compared with the BRCA2 (17.7 ± 1.8) was observed. These differences were not statistically significant ($P > 0.05$, Mann-Whitney U test), except when we compare the number of gains between BRCAX and BRCA2 ($P = 0.0031$).

The frequency and distribution of gains and losses of each group are shown in Fig. 1. In all groups, the most common changes, observed in >50% of the cases, were gain at 1q and losses at 8p21-23 and 11q22-25. Nevertheless, intergroup comparison revealed several chromosomal regions with significant differences in the frequency of involvement (Table 1). These data suggest a different pattern of genomic progression for BRCA1 and BRCA2 cases.

Building a Predictor for Classifying BRCA1 and BRCA2 Tumors. The CGH profiles of a series of 15 BRCA1 (cases 1-15; supplemental research data; Table 1) and 13 BRCA2 (cases 25-37; supplemental research data; Table 1) breast carcinomas were used to develop using random forests (see Building a Predictor) a molecular classifier, which assigns any given tumor to either the BRCA1 or the BRCA2 group.

As part of a random forests run, we obtained measures of the importance of the 63 cytogenetic and the 3 pathologic included variables. After we eliminated the variables of lower importance, the selected variables used to estimate the probability of that a given tumor to be BRCA1 or BRCA2 were the estrogen receptor status and the following six chromosomal regions: 2p11-21, 8p11-12, 12q11-21, 15q22-26, 18p, and 18q. Five of these somatic genetic imbalances also showed statistically significant differences using a Fisher's exact test (Table 1). Using the leave-one-out cross-validation of the complete variable selection process (see Building a Predictor), a score >0.5, indicating a probability of belonging to the BRCA1 class >50%, was obtained in 12 of 15 (80%) BRCA1 tumors and 5 of 13 (38%) of the BRCA2 tumors (Fig. 3A and B). To further validate the predictor, we run a new set of tumors. Nine BRCA1 (cases 16-24; supplemental research data; Table 1) and six BRCA2 (cases 38-43; Supplemental Research Data; Table 1) breast carcinomas were analyzed. The classification scores for this validation set are depicted in Fig. 3C. All the cases, except for one case of each group, were properly classified. The probability of being a BRCA1 tumor among the 43 BRCA1/BRCA2 tumors analyzed was estimated, with an accuracy of 76.7%, a sensitivity of 83.33%, and a specificity of 68.42%.

Figure 4 depicts the a posteriori probability of these regions as they are used in the predictor. These histograms represent the probability of that a tumor is BRCA1 or BRCA2 given the particular aberrations observed in the tumor and provide inside of the specific pattern of tumor progression. Therefore, the BRCA1 profile yielding the best classification performance is as follows: no change or gain at 2p11-21; no change or loss at 8p11-12 and 12q11-21; losses at 15q22-26, 18p, and 18q; and a negative estrogen receptor status. The BRCA2 profile is as follows: no change or loss at 2p11-21; no change or gain at 8p11-12, 15q22-26, 18p, and 18q; gain at 12q11-21; and a positive estrogen receptor status.

To confirm that the selected variables not exhibit statistically significant differences between the BRCA1/BRCA2 carriers with or without a recurrent mutation, we compared the profile of BRCA1 tumors del185AG (six cases) and BRCA2 tumors 3036delAAAC (three cases) versus the rest of the tumors without recurrent mutations. Only slight differences between these groups were observed (Table 2). The only variable included in the predictor and with a significant difference among the BRCA1 cases with or without the del185AG recurrent mutation was the loss of 18q.

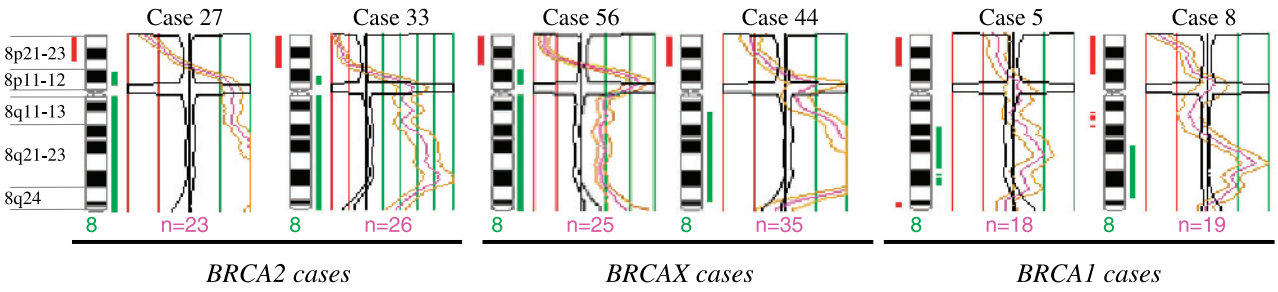

Fig. 2 Examples of HR-CGH profiles of chromosome 8 to illustrate how the genomic imbalances affect different regions within the short arm of the chromosome 8. Loss of the 8p21-23 bands are observed in all the cases. However, the 8p11-12 region shows a high heterogeneity. Gain of this region is observed on cases 27, 33 and 56 no changes on cases 44 and 5, and loss on case 8.

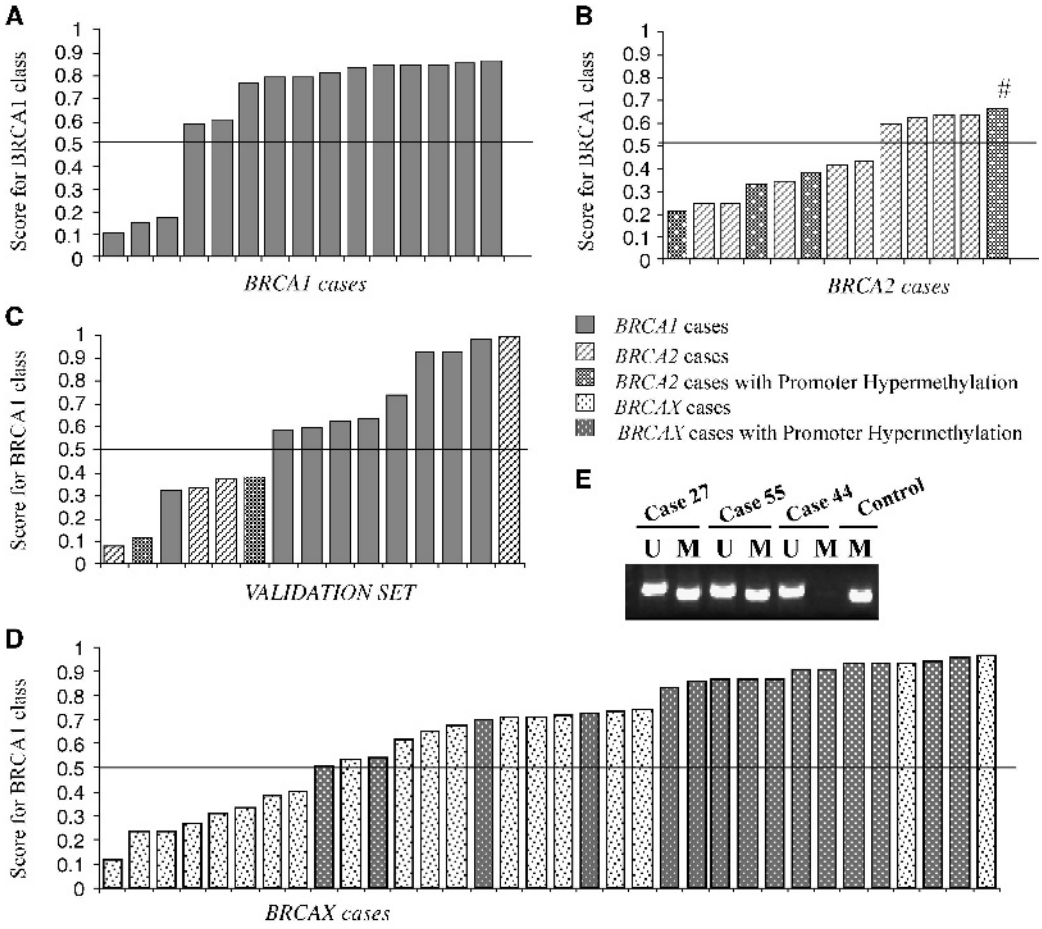

Fig. 3 BRCA1 classification score for each individual tumor. *A* and *B,* BRCA1 probability, calculated using the leave-one-out cross-validation of the complete variable selection process, of the BRCA-positive mutation cases used to build the predictor. *C* and *D,* BRCA1 probability of the BRCA-positive mutation cases of the validation set and the BRCAX cases estimated with the predictor. A high score indicates a high probability to belong to the BRCA1 class. *#,* case 34, this case cluster with the BRCA1 tumors in an ongoing expression profile study (*D*): methylation analysis of the BRCA1 promoter region in tumor samples from three patients with breast cancer. *U,* unmethylated alleles; *M,* methylated alleles. Tumors 27 and 55 present *BRCA1* promoter hypermethylation. *In vitro* methylated DNA is used as a positive control for methylated alleles.

Effect of the BRCA1 Promoter Hypermethylation on the Somatic Genetic Profile and on the Tumoral Phenotype of BRCAX and BRCA2 Tumors. Looking for similarities and differences on the BRCAX tumor profiles, we analyzed these cases using the BRCA1/BRCA2 predictor. In 76.5% of the cases, a probability of being a BRCA1 case >50% was obtained (Fig. 3D).

To determine if the high frequency of the BRCA1 pattern of tumor progression among the BRCAX and BRCA2 cases could be due to the *BRCA1* gene silencing by epigenetic factors on these two groups, we analyzed the *BRCA1* promoter region for aberrant methylation. Testing (in a blinded fashion) of all specimens of BRCAX group from our study indicated that 44% of the BRCAX tumors (15 of 34 BRCAX cases) had hypermethylation of the *BRCA1* promoter region. When we consider the predictor, we observed that all the cases with this epigenetic event were assigned to the BRCA1 class with a probability of >50%. Interestingly, 84.6% (11 of 13) of the cases assigned to the BRCA1 class with a probability of >80% had an aberrant methylation of the *BRCA1* promoter (Fig. 3D).

These results leave out whether the *BRCA1* promoter methylation events could also be occurring as germ line events. We analyzed the methylation status of the *BRCA1* promoter on eight DNA obtained from five normal breast tissues and three peripheral blood leukocytes from five BRCAX cases. All these BRCAX patients showed *BRCA1* promoter hypermethylation on the tumor sample (two of these cases, cases 68 and 73, are included on this study). All the DNA analyzed samples were found unmethylated. These data suggest that methylation is not a heritable trait on these families but a frequent event during tumor progression.

We also tested all the BRCA2 tumors and we found that 31.6% (6 of 19) of them were methylated. Among the methylated cases, only case 34 was misclassified as *BRCA1*. This case has also been classified by immunohistochemical and cDNA expression studies as a *BRCA1* tumor (data not shown).

Statistical analysis comparing the family history, the grade, and the steroid receptor status of methylated versus unmethylated BRCA2 and BRCAX tumors did not show significant differences (Fisher's exact test, $P > 0.05$).

Table 2 Chromosomal regions with a significant difference between BRCA1/BRCA2 mutation carriers used to build the predictor with recurrent and nonrecurrent mutations (Fisher's exact test, not corrected for multiple testing, $P < 0.05$)

	Percentage of tumors affected				*P*	
Regions	BRCA1 recurrent mutation (del185AG), $n = 6$	BRCA1 non-recurrent mutation, $n = 9$	BRCA2 recurrent mutation (3036delAAAC), $n = 3$	BRCA2 non-recurrent mutation, $n = 10$	BRCA1 recurrent mutation vs. BRCA1 nonrecurrent mutation	BRCA2 recurrent mutation vs. BRCA2 nonrecurrent mutation
2q35-37 Gain	50	0	33.3	0	0.046	NS
15q11-21 Gain	0	0	66.6	0	NS	0.038
14q11-23 Loss	50	0	33.3	10	0.044	NS
18q Loss	16.6	88.9	0	20	0.010	NS

DISCUSSION

Using HR-CGH, we have detected a higher number of somatic genetic changes accumulated during breast cancer development and progression in tumors of BRCA1/BRCA2 mutation carriers than in previous studies (11, 12). This novel software, due to its 2- to 3-fold improved sensitivity, has been proven to be a good system to detect chromosomal changes present in at least 50% of the analyzed cells (27, 28). This is an important feature when primary tumors that always have a variable component of normal tissue are analyzed. We found that the most frequent genetic changes, as gains at 1q and losses at 8p21-23 and 11q22-25, were present in >50% of the cases in all three patient groups. Because these abnormalities have been described also in previously reported sporadic tumors (29), they can represent a core of abnormalities common to breast cancer.

Although a similar frequency of involvement at the chromosomal regions reported previously to be significantly different between BRCA1/BRCA2 tumors and sporadic breast carcinomas were observed (supplemental research data; Table 2; refs. 11, 12), we did not find the low frequency of 16q loss and the high presence of 20q gain reported previously on the BRCA2 tumors (11). These discrepancies may be due to the differences among the studied populations, with 80% of the cases of the previously studied nordic population carrying a 999del5 BRCA2 mutation. In addition, our intergroup comparisons revealed significant differences, as gains of the 15q11-21 region, which occur in 66.6% of the BRCA2 tumors with the 3036delAAAC mutation and in none of the non-recurrent tumors (Table 2). These facts suggest that different mutations may slightly modify the profile of somatic changes along the tumor progression.

A Classifier Based on Distinct Patterns of Tumor Progression among the BRCA Mutation Carriers. Recently, the BRCA1 profile has been used to build a classifier to prescreen high-risk patients. This predictor distinguished with a high accuracy (84%) among BRCA1 and sporadic tumors. The regions used in this classifier are located at 3p, 3q, and 5q, but these

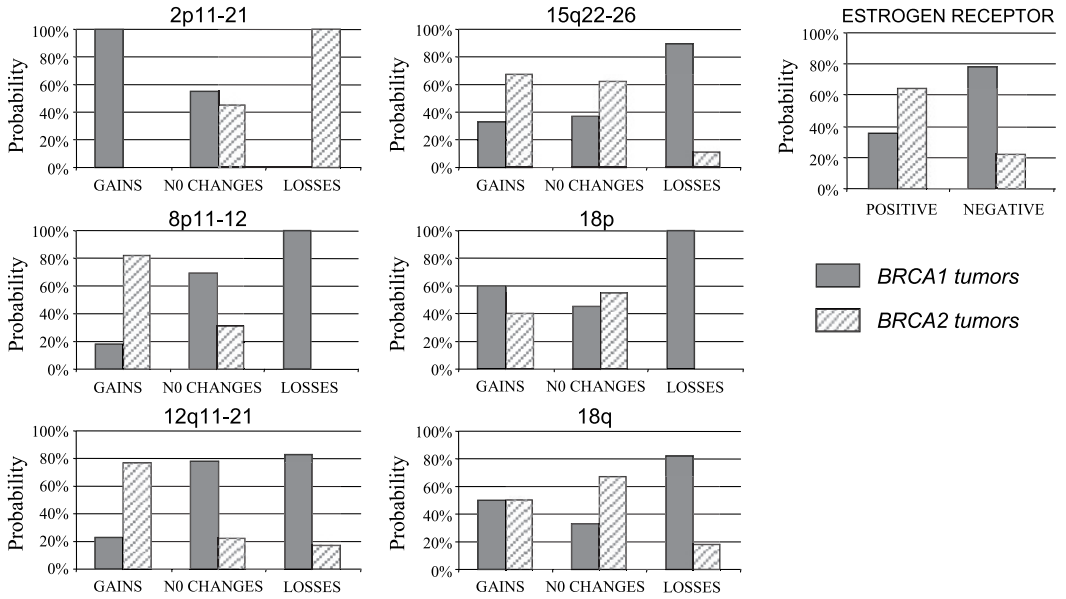

Fig. 4 A posteriori probabilities for the different variables used in the classifier. Distribution of the imbalances for the seven selected variables. The probability of a tumor to be classified as BRCA1 or BRCA2 given the particular aberration observed at a variable. For example, for a tumor with 8p11-12 gain, the a posteriori distribution indicates that the probability that the tumor belongs to the BRCA2 class is high (82%) and that is low (18%) for the BRCA1 class.

regions did not show significantly different frequencies of imbalances when BRCA2 and BRCA1 tumors are compared (12).

In our study, several chromosomal regions seemed to be preferentially involved in the BRCA1 cases and yet others in the BRCA2 cases. By using random forests, we have developed for the first time a simple and good molecular classifier based on the genomic pattern of somatic changes accumulated during the tumor development that can be used to distinguish between BRCA1 and BRCA2 mutation carriers with an accuracy of 76.7%. Previously, this supervised classification method has been used for building, with similar accuracy to other methods, good prediction models (30). We obtained a high sensitivity (83.3%) identifying the BRCA1 cases, but further studies to refine the profiles, such as CGH array and a detailed analysis of the status of the *BRCA1* gene among the BRCA2 tumors, are needed to improve the specificity (68.4%). The variables used to define the distinctive patterns of tumor progression in our classifier are six somatic genetic imbalances (2p11-21, 8p11-12, 12q11-21, 15q22-26, 18p, and 18q) and the estrogen receptor status. Some of these regions as 12q and 18q have also been found significantly more frequent lost in the BRCA1 cases than in the sporadic tumors (11, 31). Interestingly, we have shown previously using a familial breast cancer tissue microarray that the expression of the *BCL2* gene located at 18q21.3 is more frequently lost in the BRCA1 cases (89.5%) than in the BRCA2 (57.1%) and BRCAX cases (44%; ref. 10). This is in good agreement with the higher frequency of 18q loss observed by HR-CGH in the BRCA1 (60%) compared with the BRCA2 (15.4%) or the BRCAX cases (56%).

We attempted several approaches to build a classifier to distinguish the three classes (see supporting information). However, we could not define a somatic genetic profile that distinguished between the BRCA1/BRCA2 mutation carriers and the BRCAX cases. Therefore, we assumed that the pattern of tumor progression in our BRCAX cases is not quite different to the BRCA1 and BRCA2 mutation carriers, and we analyzed the BRCAX cases with the classifier. Seventy-six percent of the cases were classified as BRCA1, with a probability of >50%, confirming the somatic genetic similarities that we found by previous statistical analysis (Table 1; Fig. 3D).

Effect of DNA Methylation on the Somatic Genetic Profile of the BRCA2 and BRCAX Cases. Because *BRCA1* promoter hypermethylation was shown to silence the *BRCA1* gene in ~13% of sporadic breast carcinomas (26, 32), we studied this phenomenon as a mechanism of *BRCA1* silencing in the BRCAX and the BRCA2 cases. We found this epigenetic event in 44% of the BRCAX (15 of 34) and in 31% of the BRCA2 cases (6 of 19). Among the BRCAX cases, 84.6% of the cases (11 of 13) assigned with a probability of >80% to the BRCA1 class were *BRCA1* promoter hypermethylated (Fig. 3D). This epigenetic phenomenon was not observed at the germ line level.

BRCA1 does indeed seem to be implicated in sporadic breast and ovarian tumors. Aberrant methylation of *BRCA1* seems to be a crucial player in the development of these tumors. Already, some genomic changes as MYC amplification have been found in a significantly higher proportion on sporadic tumors with *BRCA1* dysfunction (33). In addition, it has been suggested that *BRCA1* hypermethylation could influence the phenotype of the tumor on a similar way to a genetic mutation (34). Our study suggest for the first time that this epigenetic event could also influence the tumor progression genotype on a similar way to a genetic mutation in most of the BRCAX cases. Further clinical studies will be needed to know if the selection of these patients is clinically relevant.

We have not found statistically significant differences in the family history or in the steroid receptor status between methylated and unmethylated tumors. However, in the analysis of a tissue array of BRCAX cases, a statistically significant higher percentage of p53 and Ki-67 positivity has been observed in the *BRCA1* methylated tumors, indicating a higher proliferation rate and suggesting a more aggressive behavior of these cases.[7]

The identification of a method to successfully subdivide the histopathologically heterogeneous group of BRCAX families into recognizable groups could be of considerable value to further genetic analysis. Previously, global gene expression analysis have discover novel classes among BRCAX tumors (35). Our findings illustrate that also at the genomic and epigenetic levels BRCAX families can be grouped into homogeneous subsets, thereby potentially increasing the power of genetic linkage analysis. Moreover, the complete sequencing of the *BRCA* genes is very difficult due to the large number of exons of these genes; this tool could allow us to select cases among the BRCAX patients for further genetic studies as large deletions or rearrangement analysis. At the therapeutic level, these data could point to the inclusion of demethylating agents into the treatment protocols for familial breast cancer.

Among the six BRCA2 cases with *BRCA1* promoter hypermethylation, five cases were correctly assigned to the BRCA2 class and only case 34 was assigned to the BRCA1 class. This case also clusters with the BRCA1 tumors in ongoing expression studies from our laboratory (Fig. 3B). These data suggest that hypermethylation of the *BRCA1* gene in the majority of the BRCA2 tumors could be a later event in the genetic progression, when the specific genetic pathway is yet established.

In summary, we have build a simple molecular classifier that distinguish with an accuracy of 76.7% between BRCA1 and BRCA2 tumors. This BRCA1 pattern of tumor progression is frequently found among the BRCAX tumors and, in more than half of these cases, is associated with *BRCA1* promoter hypermethylation, suggesting that this event can modify the profile of tumor development in most of the BRCAX tumors. Further studies to better establish the genomic integrity of the *BRCA1* gene are guaranteed. Finally, the presence of this epigenetic event among the BRCA2 cases is also frequent but not associated in the majority of the cases with a BRCA1 profile.

ACKNOWLEDGMENTS

We thank the Spanish Tumor Bank Network and the Inmunohistological Unit of the Spanish National Cancer Center for technical support, Adele Cutler and Andy Liaw for comments on variable selection for random forests, and Ellen Allacid for assistance in preparing the article.

[7] Honrado et al., unpublished data.

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Analysis of Myelodysplastic Syndromes with Complex Karyotypes by High-Resolution Comparative Genomic Hybridization and Subtelomeric CGH Array

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Molecular cytogenetic techniques enabled us to clarify numerical and structural alterations previously detected by conventional cytogenetic techniques in 37 patients who had myelodysplastic syndromes with complex karyotypes. Using high-resolution comparative genomic hybridization (HR-CGH), we found the most recurrent alterations to be deletion of 5q (70%), 18q (35%), 7q (32%), 11q (30%), and 20q (24%), gain of 11q (35%) and 8q (24%), and trisomy of chromosome 8 (19%). Furthermore, in 35% of the patients, 20 amplifications were identified. These amplifications were shown by FISH to involve some genes previously described as amplified in hematological malignancies, such as *ERBB2*, *MLL*, and *RUNX1*. In addition, two other genes, *BCL6* and *BCL2*, which are classically related to apoptosis and non-Hodgkin lymphoma, were shown for the first time to be involved in amplification. Genomic alterations involving different subtelomeric regions with losses in 4p16, 5p15.3, 6q27, 18p11.3, and 18q23 and gains in 1p36.3 and 19p13.3 were detected by HR-CGH. Array CGH analysis of the subtelomeric regions in some samples was able to confirm a number of these alterations and found some additional alterations not detected by conventional CGH.

INTRODUCTION

Cytogenetic analysis provides essential information about the importance of diagnosis and prognosis in patients with hematologic malignancies (Grimwade et al., 1998; Rowley, 1998, 1999). At present, cytogenetic analysis using G or R banding is the most widely used method for the identification of chromosome aberrations in leukemic cells, although interpretation of the banding patterns is often complicated by chromosome condensation, imperfect banding, and having few metaphase cells. These limitations are evident in complex karyotypes (CKs), those that have three or more abnormalities, and, thus, it is often impossible to identify the chromosomes and/or chromosome bands involved in balanced or unbalanced chromosomal rearrangements.

CKs are common in patients with myelodysplastic syndromes (MDSs), 15%–30% of whom carry a CK at diagnosis, and specific alterations are rare (Fenaux et al., 1996). These MDSs tend to progress to acute myeloid leukemia (AML) and confer a poor prognosis (Greenberg et al., 1997). Therefore, identification of specific alterations that could define a subset of patients with this disease, or even contribute to the identification of the genes involved, could provide targets for the development of new therapeutic approaches for these patients. Therefore, improved characterization and a comprehensive description of CKs are essential. New molecular cytogenetic techniques such as comparative genomic hybridization (CGH), spectral karyotyping (SKY), and fluorescence in situ hybridization (FISH) allow better identification of genetic gain and loss in the tumor genome and permit the characterization of new alterations. However, as yet, only a small series of patients with MDS with a CK have been analyzed by CGH and/or SKY as reported in the literature (Kakazu et al., 1999; Kim et al., 2001; Lindvall et al., 2001; Verdorfer et al., 2001; Mrozek et al., 2002; Van Limbergen et al., 2002). In previous work

Supported by: Spanish Ministry of Science and Technology; Grant number: SAF-01-0056; Red Temática de Centros del Cáncer, Spanish Ministry of Health; Grant number: CO-010; Instituto de Salud Carlos III (to A.M.R).

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Received 6 July 2004; Accepted 20 September 2004

DOI 10.1002/gcc.20154

Published online 20 December 2004 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Patients' Characteristics and Cytogenetic Description of their Karyotypes

Case	Age	Type	G-band karyotype	SKY karyotype*
1*	54	RAEB	45,XY,-5/44,XY,-5,-17,-18, +mar/46,XY	45,XY,-5[2]/44.XY,-5,der(17)t(5;17)(¿;p11) hsr(17)(q21,22),-18[13]/46,XY[3]
2*	81	RAEB-T	47,XY,+8,del(11)(q23),del(20)(q12)/ 46,idem,-Y	47,XY,+8,t(11;20)(q23;q11)[12]/46,X,-Y, +8,t(11;20)(q23;q11)[3]
3*	77	RAEB-T	43,X,-Y,del(5)(q13q33),-7,der(7)(?), add(8)(q24),-13,-16,-18,add(20)(q13), -21,+3mar/46, XY	44,XY,del(5)(q13),-7,der(7)hsr(7)(p?)t(7;18) (q25;?),der(16)t(16;18)(q12;?),-18,del(20) (p12)[11]/44,idem,der(20)t(9;20)(?;p12)[5]
4*	69	RAEB-T	45,XY,del(11)(q23),-12,del(18)(q12), del(20)(q13)	45,XY,del(7)(p15p21),der(11)t(11;18) (p11.2;q11.2),der(12)del(12)(p11.2)del(12) (q15qter),del(16)(q22),del(18)(q12),-20[9]
5*	60	RAEB-T	46,XX,t(4;11)(q32;p12),del(5)(q15q35), add(13)(p13),-18,-20,-21,+3mar	46,XX,der(4)t(4;5)(q27;?),del(5)(q13), der(11)t(4;11)(?;p14-15),-13,der(17)t(11;17) (?;p11.2),der(18)t(13;18)(q12;p11.2)[10]/ 46,X,t(X;20)(p11.4;q11.2),idem,+20[3]/46,XX(2]
6*	75	RAEB-T	54,XX,+1,+2,+3,del(5)(q13q33),+6,+8, +14,add(19q),+21,+2mar/46,XX	46;XX,del(5)(q14q34)[2]/54,XX,+1, +2,del(5)(q14q34),+6,+8,+13,+14,+19,hsr(19) (p?),del(20)(p11.2),+21[6]/46,XX(8]
7*	74	RAEB-T	47,XX,+8,-20,i(21)(q10),+del(22)(q11)/ 46,XX	47,XX,+8,del(11)(q23),der(20)t(20;21) (q11.2;q11.2),-21,+mar[8]/46,XX[4]
8*	74	RAEB	46,XY,+del(1)(p22),del(5)(q13q33), -6,der(11)(q?)/45,XY,idem,-22	46,XY,del(5)(q13q33),del(11)(q13), der(22)t(11;22)(q12;p11),der(11)t(11;22) (p11;q11)hsr(11)(q23),der(11)del(11)(p?) del(11)(q?)[11]/45,idem,-7,der(18)t(7;18) (?;q11.2)[5]
9*	71	RAEB-T	47,XY,del(5)(q13q33),+21/48,XY, del(5)(q13;q33),+der(21)(?),t(8;21) (q22;q22),+2mar	46,XY,del(5)(q21)[5]/48,XY,del(5)(q21), +dup(21)(q21),+r(21)(p?q?)[9]/ 48,idem,del(17)(p11.2)[5]
10*	69	RAEB	45,XY,t(3;3)(p25;q23),t(6;21) (p21;q22),-7	45,XY,t(6;21;22)(p21;q22;q13),-7[7]/ 45,idem,t(3;3)(p25;q23),del(17)(p11.2)[7]
11*	76	RAEB	46,XX,del(5)(q13q32),del(6)(q22), add(8)(q24)	46,XX,del(5)(q14q34),del(6)(q22), ins(8;6)(q22;?)[8]
12*	63	RA	46,XX,i(17)(q10)/46,XX,+X, -9,i(17)(q10)/46,XX	46,XX,del(17)(p11.2)[2]/46,XX,i(17)(q10)[5]/ 46,XX,idem,del(19)(p?)[9]/46,XX[2]
13*	67	RAEB-T	44,XX,-2,add(3)(p26),-5,add(6)(p25), +8,add(11)(q25),-13,-13,-15,-18, add(17)(p13),-22,-22,+4mar/46,XX	45,XX,-2,+5,der(5)t(5;6)(q13;?),der(6)t(2;6) (?;p25),+8,der(11)t(11;16)(q21;?), +der(11)t(11;15)(q13;q15), -13,der(13)t(13;13)(q34;q12),-15,-16, der(17)t(2;17)(?;p11.2),der(22)t(16;22)(?;p12)[4]/ 46,idem,+19[5]/44,idem,der(3)t(3;5)(p25;?),-5[4]/ 43,idem,-8,-18,der(7)t(7;17)(p11.2;q11.2), del(17)(q12)[2]/46,XX[5]
14*	67	RAEB-T	45,XX,t(7;21)(q10;p10),-21/44,XX, idem,-5,add(17)(p13)/46,XX	44,XX,-5,der(7)t(7;21)(p10;q10),del(11)(q?), der(17)t(5;17)(?;p11.2),-21[10]/46,XX[2]
15*	67	RAEB	42,XY,del(3)(q13),der(3)(q?), -7,add(9)(q34),-11,add(12)(p13), -14,-18,-22,+mar	46,XY,der(3)t(3;18)(q11.2;q21),del(5)(q13), der(9)t(3;9)(q21;q34),del(11)(q13),der(18) del(18)(q21)[6]/44,XY,der(3)t(3;18)(q11.2;q21), der(5)t(5;14)(q13;q13),der(9)t(3;9)(q21;q34), del(11)(q13),-14,-18[4]/43,idem,-7[4]/43, idem,-7,l(22)(q10)[3]/41,idem,-7, -del(11)(q13),der(16)t(16;17)(q12;q11.2),-17[3]
16*	78	RAEB-T	47,X,-X,del(3)(p?),del(5)(q13q33),+6, -7,-10,+14,add(16)(p13),add(19) (q13),add(21)(q22),+2mar/46,XX	46,XX,del(5)(q21)[5]/46,X,t(X;2)(p22.1;p11.2), der(2)t(2;19)(p11.2;q13?),del(4)(q22),del(5)(q21), der(16)t(5;16)(?;q12)[8]/46,XX[3]
17*	84	RAEB-T	46,XY,der(3)(q?),del(5)(q?),der(12)(p?)	46,XY,del(3)(q?),del(5)(q13),del(12)(p12)[10]
18*	52	RAEB	44-45,de1(5)(q?),-20,+mar/46,XY	44-45,XY,del(5)(q11.2),dup(16)(q?),der(17) t(5;17)(?;p11.2),-19,-20[6]/44-45,idem, der(17)t(17;20)(p11.2;q?)[11]
19*	64	RAEB-T	47,XY,del(7)(q?),del(20)(q?), der(22?)t(9;22)(q34;q11),+mar/46,XY	46,XY,del(7)(q11.2),del(20)(q11.2)[16]/46,XY[5]

(Continued)

TABLE 1. Patients' Characteristics and Cytogenetic Description of their Karyotypes (Continued)

Case	Age	Type	G-Band karyotype	SKY karyotype*
20*	64	RAEB	48,XY,del(20)(q?),+2mar	46,XY,del(17)(p11.2),del(20)(q11.2)[9]/46,XY[3]
21*	64	RAEB-T	45-46,XX,del(3)(q21q26), -4,del(5)(q13q35),+del(8)(q22)?, del(11)(q23?),del(12)(p13), add(13?)(q32?),+15,i(17)(q),+18,-20, +add(21)(q22),del(22)(q11?),+mar	46,X,t(X;20)(p11.4;q11.2),del(3)(q25),t(4;13) (q26;q13)inv(4)(p16q26),del(5)(q15q32), -7,+8,del(9)(p13p21),del(11)(q22),del(12) (p12pter), der(16)t(7;16)(p14;q12)[16]/45,idem, der(9)t(9;22)(q34;q11.2),-22[3]
22*	78	RAEB-T	48,X,-X,+8,+2mar	48,XX,+idic(X)(q13)x2[3]/48,X,-X, +idic(X)(q13)x3[6]/48,X,-X, +idic(X)(q13)x2,+8[4]
23*	72	RAEB	46,XY,del(11)(q?)/52-54, Complex Karyotype (not defined by G-banding)	51-53,Y,t(X;10)(p11.4;q24),+del(5)(q13), +del(5)(q23),der(6)del(6)(p11.2) del(6)(q11.2)x2,-11, der(11)hsr(11)(q23),+15,+16, +der(20)hsr(20)(q?)[11]/46;XY[5]
24	66	RAEB	44-45,XY,del(3)(p?),t(5;11)(q?;?),-17	ND
25	70	RAEB	46-52,Complex Karyotype (not defined by G-banding)	ND
26	67	RAS	46,XY,del(5)(q13q35), del(10)(?),i(17)(q?)	ND
27	71	RAEB	47-49 Complex Karyotype (not defined by G-banding)	ND
28	88	RAEB-T	54-55 Complex Karyotype (not defined by G-banding)	ND
29	76	RAEB	47-50 Complex Karyotype (not defined by G-banding)	ND
30	81	RA	52-54 Complex Karyotype (not defined by G-banding)	ND
31	70	RAEB	47-50 Complex Karyotype (not defined by G-banding)	ND
32	65	RAEB-T	47,X,der(X)t(X;18?),del(5)(q13q33), t(5;11;12)(q?;q?;p?),r(7),del(18)(q?), +mar	ND
33	69	RA	49,XX,+X,t(1;3)(q32;q21),+11,-18, +2mar/46,XX	ND
34	56	RAEB-T	83-84,XXXX,del(3)(q23),del(5) (q13q33),del(17)(p12)x2/46,XX	ND
35	64	RAEB	44,XY,-5,-7,i(10)(q10),-13, der(16)(q),der(17)(q10), -18,+2mar/46,XY	ND
36	71	RAEB	50-52,XX,i(11)(q10)x2, Complex Karyotype (not defined by G-banding)	ND
37	80	RAEB-T	46,XY,del(1)(p32),-5,-7,+8,add(12) (p13),-18,-19,-20,+4mar/46,XY	ND

*SKY karyotypes previously published (Martinez-Ramirez et al., 2004). Numbers in parentheses refer to number of analyzed metaphases; ND, not done.

(Martínez-Ramírez et al., 2004), we studied 23 patients who had MDS and CK by SKY, and we found some specific alterations, including two recurrent translocations, der(17)t(5;17) and t(X;20), total or partial loss of the *TP53* gene in 25% of cases, and homogeneously staining regions with mild amplification levels (more than 10 copies) involving the *ERBB2*, *MLL*, and *RUNX1* genes. These descriptions contribute to a better definition of the cytogenetic profile of these cases. In the present study, we extended our analysis by using high-resolution CGH (HR-CGH) in a larger series of cases of MDS with CKs. We confirmed our previous results, and we found amplifications of other genes not previously related to these types of disorders, such as *BCL2* and *BCL6*.

MATERIALS AND METHODS

Patients

Thirty-seven patients were selected at diagnosis from two centers in Spain: the Spanish National

TABLE 2. Gain and Loss Detected by HR-CGH (version 2.72)

Case	Alterations detected
1	rev ish enh(7q22,11q13),dim(3p23p24.5,5q11q33,6q23qter,17q23qter,18(x2),Xq25,Xq27),amp(17q11q22)
2	rev ish enh(8(x2),10q11.2,16q13q21), dim(7p21,10p12,10p21,11q23,12q21,12q24.1,13q21,13q31,20q12q13.1,Y,Xp22.3)
3	rev ish enh(3p24p25,7q22,11q13,19p13.3),dim(6q27,11q14q22,12q24,1qter,16q23,18p11.3,20p12pter)
4	rev ish enh(21), dim(2p21p22,5p15.3,5q34,6q26,7p15p21,8p23,11q14q22,12,16q22qter,18p11.3,18q12,20p,q13.2)
5	rev ish enh(21q21,22q11.2), dim(3q27qter,4q34q35,5q14q33,17p11pter,18p11pter,20q11q13.2), amp(20p11pter)
6	rev ish enh(1,2,6,8,19p12pter), dim(3q27qter,4.5p15.1p15.3,5q14q33,7p11pter,12p12,18q12,18q22,20p12pter,Xp27)
7	rev ish enh(8,11q14), dim(3q26.3qter,11q23,18p11.3,20q11qter,21q21)
8	rev ish enh(1p31,1q22q31,2q22,3p21,4q11q27,6q12q22,13q21,22), dim(5q14q33,18q21q22), amp(11q14qter)
9	rev ish enh(1p22p31,1q23,6q12q14,11q14q22,12q21,21), dim(5q23q33,6p23pter), amp(21q11q21)
10	rev ish enh(8q23,10p12,21q22), dim(5q32,6p22,7(x2))
11	rev ish enh(12q15q21,13q21q22), dim(5q21q33,6q25)
12	rev ish enh(1p36.3,4q28,17q11qter,22q13), dim(4p16,4q31q33,6p22,7p21,16q22q23,17p11pter,Xq25q27)
13	rev ish enh(6p21.1p21.3,11q23,13q32qter,19q13.2), dim(2p14.2q24,7p11pter,8p23,9p21p22,11p15,11q22, 18q11qter,Xq21,Xq27qter)
14	rev ish enh(19p13.1), dim(5q11qter,7p11pter)
15	rev ish enh(1q32,3q25qter,9q31qter,10q24,11q13,12q12q13,17q12qter,19p13.3,22q12q13,Xq24), dim(2p24pter,3q12q13.3,5q14qter.7,11p14,11q14q22,12p13,18p-q21,18q22qter), amp(3q27q29)
16	rev ish enh(8q22q23,21q22), dim(3p21p24,4p16,5p15.3,5q21q33,7p14,9p23,10q24,18q23)
17	rev ish dim(3p24pter,3p14,3q13.2q24,5p15.3,5q14q33,6p25,6q27,7p14,8p22pter,11q14q22,11q24qter,12p12,12q21, 13q21qter,18q12q22,Xq28)
18	rev ish enh(1q32,7q11.2,7q22,12q12q13,17q21,19p13.3), dim(4p16,5q14qter(X2),10q26,17p11pter,20p12,20q11.2q12)
19	rev ish enh(12q21q23,17q11.2q21), dim(2p22,4p15.3p16,5p15.1p15.3,5q34qter,7p21,7q21qter,11q22,18q11qter, 20p12.20q11qter,21q22,Xq21)
20	rev ish enh(1p36.3), dim(1p31,2p25,4q13q33,5q33,7p14,7q31,9p23,14q24,20p12,20q12q13.2.Y,Xq25)
21	rev ish enh(1p12p13,2q32,5q11.1q13,8(x2),12q13,17q23qter), dim(3q28,5q14q33,7q11qter,9p21,11q21,12p12,16q21qter)
22	rev ish enh(1q21,8,16p12), dim(4p16,5p15.3,6q27,7q36,18q23,Xq13qter), amp(Xq13-pter)
23	rev ish enh(6p21.1p21.3,8q23,11q22q23,Xq21), dim(3p25,10q25,12p12)
24	rev ish enh(3q21q25,4q12q31.1,8q11q13,8q21q23,11,17q21q22,Y), dim(3p13pter,5q11q33,6q25,18q23)
25	rev ish enh(1q31,4p16,5q34,6p21.1p21.2,8q24,9p24,9q34,20q11qter,22q11.2qter,X(x2)), dim(4q22q27,5q14q33,7q22qter,18p11.3,18q22qter), amp(18q11.2,18q12q22,21q22)
26	rev ish dim(5q15q33,6q27,8p22pter,10q21q25,13q31,17p12,19p13.2,Xp21,Xq22)
27	rev ish enh(1q31,4q22q24,9p21p22,9q34), dim(5q21q23,6q25qter,7q32qter,8p22,10p15,18p11.3,20q13,Xp22.1,Xq27), amp(Y)
28	rev ish enh(1,4q28q33,6,8,10,11,13,14,19,21q22), dim(3q29,5q14q22,7p21,7q21q35,9p23pter,17q23q24)
29	rev ish enh(9q12qter,19,), dim(5q11qter,7q21qter,8p22pter,9p11pter,10,11,12p12,13,14,15,16q11qter,18q11qter,X), amp(21q22,Y)
30	rev ish enh(10q22,11q14q21,19q13.2), dim(5p15.2p15.3,7p22,20p12,20q13)
31	rev ish enh(3,14q23q24,15q22qter), dim(4q22q24,6p22pter,7q22q35,8p11pter,9q21q22,15q21,Xp-q26), amp(8q22q24, 18q12q21,18q22)
32	rev ish enh(1p12p13,6p21.3,8q21.1,8q22,8q23,9q34,11q13,22q13), dim(5q14q33,7q31qter,8p22,10p12,11q14q22, 12p12,18q12), amp(11q23qter,20p12p13?)
33	rev ish enh(8q21.3q22,11p15), dim(3q27qter)
34	rev ish enh(1q23q31,2q22q23,4q26q28,5p14,8q21,8q23,11q14q22,13q21q31,14q13,22q11), dim(2q35qter,3q21q23,3q27qter,5q13q33,5q35,10p12,12p11pter,16q21q23,17p11pter,18,Xp22.1p22.2)
35	rev ish enh(3q25q27,4q22q23,4q26,4q28,5q15q23,6q11qter,11q14q22,Xq24), dim(3p25,7p11pter,10p14,17p11pter), amp(3q26q27,21q22)
36	rev ish enh(1,5p-q21,5q33qter,12q14.22), dim(2q13q21,5q21q23,7q21,13q22,Xp22.3,Xp21,Xq23), amp(11q12qter)
37	rev ish enh(1p33p34,8,11,12p13,19p13.2,19q13.3), dim(1p21p22,5q11q33,7q11qter,16q22qter,17p11pter)

rev ish: reversed in situ hybridization; enh: enhanced; dim: diminished; amp: amplified.

Cancer Center (CNIO) and the University of Navarra. Selection criteria were: diagnosis of MDSs according to the FAB criteria (Bennett et al., 1984) and the presence of three or more chromosomal aberrations at diagnosis. The group consisted of 20 men and 17 women, with a median age of 70 years (ranging from 54 to 84 years). All but three patients had RAEB and RAEB-t subtypes. A summary of the patients' characteristics is shown in Table 1.

Conventional Cytogenetics

Bone marrow samples were incubated in RPMI 1640 with 20% fetal calf serum for 1 day at 37°C. Cells were exposed to Colcemid (0.1 μg/ml) for 1.5 hr at 37°C and harvested. Metaphase chromo-

somes were GTG-banded by a conventional Trypsin-Giemsa technique. Karyotypes were described following the recommendations of the International System for Human Cytogenetic Nomenclature (Mitelman, 1995). Cases 1–23 were published previously (Martínez-Ramírez et al., 2004).

Comparative Genomic Hybridization (CGH)

CGH was performed according to Kallioniemi et al. (1992), with some modifications. Tumor (test) and normal (reference) DNA were labeled using a nick translation kit from Vysis (Downers Grove, IL). Sex-matched references were used to perform CGH for the patients and the normal and altered controls. In short, 200 ng of each labeled DNA was hybridized to normal male metaphase cells in the presence of 20–35 μg of Cot-1 DNA for 3 days. After washes, the chromosomes were counterstained with DAPI in an antifade solution.

Digital Image Analysis and Interpretation of HR-CGH Results

Analysis was performed with an Olympus AX60 epifluorescence microscope equipped with a Sensys charge-coupled device camera (Photometrics, Tucson, AZ). A minimum of 15 metaphase cells per hybridization per case were analyzed by use of the CytoVision System with version 2.72 high-resolution CGH analysis software (Applied Imaging, Newcastle, UK). The CGH profiles were compared to a dynamic standard reference interval on the basis of an average of normal cases, as described by Kirchhoff et al. (1998). Briefly, along the mean ratio profiles, the 99.5% confidence interval of each mean ratio profile value was compared to a corresponding 99.5% standard reference interval based on an average of normal cases. The dynamic standard reference intervals are wide at regions known to produce unreliable CGH profiles. Chromosome regions in which no overlap existed between the two intervals were designated as being altered. The standard reference interval was scaled automatically to fit the individual test case.

Array CGH

The subtelomere array consisted of 414 autosomal BACs, all of which originated within 5 Mb of the telomere of each of the chromosome arms, 18 X-chromosome-specific BACs, and 3 Y-chromosome-specific BACs. For positioning the BACs with respect to the sequence of the human genome, we used the July 2003 human reference sequence (UCSC version hg16) based on NCBI Build 34.

BAC DNA was amplified by DOP-PCR (Fiegler et al., 2003) and spotted in quadruplicate onto epoxy-coated slides (Schott Nexterion, Mainz, Germany), with the use of a MicroGrid II arrayer (Biorobotics, Cambridge, UK). Array slides were pretreated prior to hybridization according to the manufacturer's protocol. Prehybridization with 2.2 μg/μl herring sperm DNA in Schott Nexterion hybridization buffer was performed at 65°C for 2 hr. Excess prehybridization solution was removed by rinsing in distilled water, and the slides were dried by centrifugation.

From 0.5 to 1.0 μg of patient tumor DNA and reference genomic DNA, consisting of a pool of 17 normal male or female DNA samples, was labeled with Cy3 and Cy5, respectively, by use of Invitrogen's (Paisley, UK)BioPrime random labeling kit. After overnight incubation at 37°C, unincorporated nucleotides were removed by use of MicroSpin G-50 spin columns (Amersham Biosciences; Piscataway, NJ). Tumor and reference DNA were combined on a microcon YM30 column and subsequently dissolved in 20 μl of 4 μg/μl of human COT-1 DNA and 20 μg/μl of yeast tRNA in Schott Nexterion hybridization buffer. Labeled DNA was denatured at 99°C for 10 min and stored at 65°C prior to hybridization. Hybridization was performed under coverslips in humidified Corning Hybridization Chambers (Corning, Acton, MA) at 65°C in a water bath for 40 hr. Posthybridization washes included a wash in 2× SSC/0.2%SDS at 65°C for 15 min, followed by a wash in 2× SSC at room temperature for 5 min, and a wash in 0.2× SSC, also at room temperature, for 5 min. Slides were rinsed briefly in distilled water and dried immediately by centrifugation.

Image Analysis and Processing

Arrays were scanned using Agilent DNA Microarray Scanner, Model G2565BA (Agilent Technologies, Palo Alto, CA). The resulting images were analyzed with GenePix Pro 5.0 software (Axon Instruments, Inc., Foster City, CA). Data were further processed with specifically designed data analysis software.

Briefly, spots were eliminated if the absolute intensity of the reference (normal DNA) sample was less than 2 times the average signal of a set of control spots consisting of *Drosophila* DNA. Raw tumor/reference ratios were calculated for all spots without background subtraction. Normalization was carried out for each subarray separately, assuming a median ratio of 1 for all spots. A spot was eliminated if it differed by more than 20% from the median

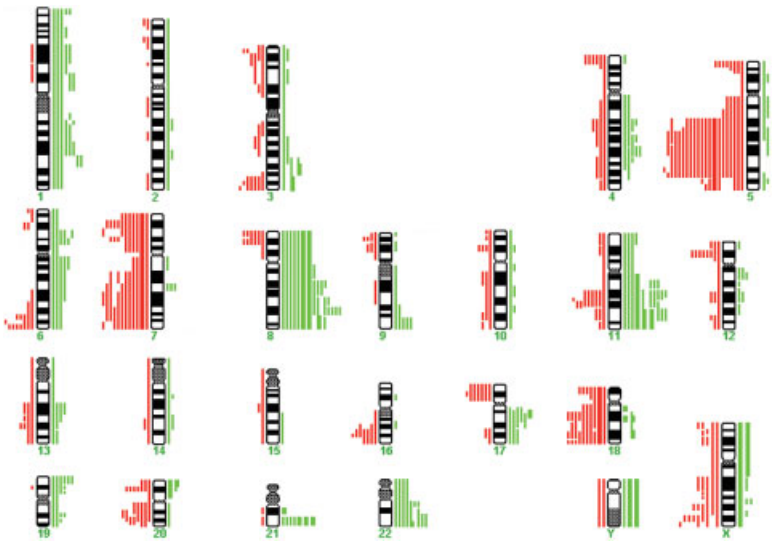

Figure 1. Distribution of chromosomal imbalances caused by unbalanced structural aberrations or numerical changes detected by CGH. To the right, gain of chromosomal material is in green, with high-level gains and trisomy shown as bold lines; to the left, loss of chromosomal material is in red, with nullisomy shown as bold lines.

ratio of all replicated spots containing the same BAC. BACs for which only one data point (spot) was left were also eliminated. For the remaining replicate spots of the BACs, the average ratio was then calculated. An analysis was rejected completely if less than 50% of all BACs met the above-mentioned criteria or if the standard deviation of all BACs was larger than 0.25. Gain or loss was assigned only if the ratios of at least two consecutive BACs on the array deviated from the normal ratio.

Fluorescence In Situ Hybridization (FISH)

Metaphase chromosomes were prepared directly from the same samples used for conventional analysis and left overnight at room temperature. Slides were placed on a hot plate at 90°C for 10 min, dehydrated through a series of ethanol washes, and denatured in the presence of a probe on a plate at 75°C for 1 min. For detection of gene amplification, gene-specific probes for *MYC*, *MLL*, *ERBB2*, *BCL2*, *BCL6*, and *ETV6/RUNX1* (Vysis, Downers Grove, IL) were used. At least 100 interphase nuclei were analyzed in cases that showed amplification and deletion. We considered a gene to be amplified when more than 10 copies were seen in the FISH analysis. Cell images were captured with a Sensys charge-coupled device camera (Photometrics, Tucson, AZ) connected to a PC running the CytoVision image analysis system (Applied Imaging, Newcastle, UK).

RESULTS

Cytogenetic Analysis by G-Banding

The G-banded karyotypes of the 37 patients analyzed are shown in Table 1 [we previously reported cases 1–23 (Martínez-Ramírez et al., 2004)]. A hyperdiploid karyotype was found in 18 patients (48.5%) and a hypodiploid karyotype in 12 patients (32.5%); the other 7 patients had a normal complement of 46 chromosomes (19%). We observed monosomies in 20 cases. Most frequently lost were chromosomes 18 (27.5), 7 (24%), 20 (17%), and 5 (14%). In 10 of the 37 cases, we detected extra chromosomes; 5 of them showed trisomy of chromosome 8 (17%). Ten reciprocal translocations were detected by G-banding in nine patients; however, the majority of structural aberrations were unbalanced (69 structural alterations).

Case 9 with a t(8;21) did not present rearrangement for *RUNX1/ETO* by FISH. Case 18 showed a t(9;22), but we could not perform FISH analysis to study the possible *BRC/ABL* fusion.

Unbalanced alterations predominantly resulted in the loss of chromosome material (40 of 69). Segments from chromosome arms 5q (48%), 11q (10%), 20q (10%), and 3q (10%) were most frequently identified as lost. Unbalanced rearrangements, resulting in additional chromosomal material of unknown origin, were observed in 10 patients, with extra material found in 8q (7%), 19q (7%), 21q (7%), and 17p (7%). We also detected seven isochromosomes of the long arms of chromosomes 17 (10%), 11 (7%), 10 (3.5%), and 21 (3.5%). Furthermore, we observed 31 unidentified marker chromosomes in 11 of the 37 patients.

Cytogenetic Analysis by HR-CGH

HR-CGH results obtained for the 37 patients are shown in Table 2 and Figure 1. We identified 434 chromosomal regions with changes, and all

TABLE 3. Summary of Main HR-CGH Findings

Summary of CGH alterations					
Losses		*60%*	*Gains*		*40%*
Whole chromosomes:			*Whole chromosomes*		
Chr. 7	(2/37)	5.5 %	Chr. 1	(3/37)	8 %
Chr. 18	(2/37)	5.5 %	Chr. 6	(2/37)	5.5 %
			Chr. 8	(7/37)	19 %
			Chr. 11	(3/37)	8 %
			Chr. 19	(2/37)	5.5 %
			Chr. 21	(2/37)	5.5 %
			Chr. 22	(2/37)	5.5 %
Partial loss			*Partial gain*		
3q	(10/37)	27 %	4q	(10/37)	27 %
5q	(26/37)	70 %			
7p	(13/37)	35 %			
7q	(12/37)	32.5 %			
11q	(11/37)	30 %			
12p	(8/37)	21.5 %	8q	(9/37)	24 %
16q	(7/37)	19 %	11q	(13/37)	35 %
17p	(7/37)	19 %	19p	(6/37)	16 %
18q	(13/37)	35 %	21q	(7/37)	19 %
20p	(7/37)	19 %	22q	(6/37)	16 %
20q	(9/37)	24 %			
Telomeric loss			*Telomeric gain*		
4p16	(4/37)	11 %	1p36.3	(2/37)	5.5 %
5p15.3	(4/37)	11 %	19p13.3	(3/37)	8 %
6q27	(4/37)	11 %			
8p23	(2/37)	5.5 %			
18p11.3	(4/37)	11 %			
18q23	(3/37)	8 %			
			High-level gain		
			3q	(2/37)	5.5 %
			11q	(3/37)	8 %
			18q	(2/37)	5.5 %
			20p	(2/37)	5.5 %
			21q	(4/37)	11 %
			Y	(2/37)	5.5 %

patients were affected by gains and/or losses. Loss of material proved to be more frequent than gain (60% vs. 40%), with 11 the median number of alterations (ranging between 4 and 20). A summary of the main alterations is given in Table 3.

Twenty-six patients had a deletion on 5q (70%), which was the most frequent alteration in this group of patients. Partial loss of 7q and 20q was identified in 12 (32.5%) and 9 cases (24%), respectively. Several cases showed partial loss of 5q combined with loss on other chromosomes, especially on 7q and 20q. Loss of whole chromosomes 5 and 7 and an apparent double loss of chromosome 7 (case 10) each were detected in one patient. However, the double loss was discarded by SKY. Gain of whole chromosome 8 was identified in seven patients (19%), two of whom (cases 2 and 21) had two additional copies. In another nine cases (24%), we detected gain of part of the long arm of this chromosome. In all patients with additional chromosome 8 material, either trisomy or gain of 8q, we also found other alteration characteristics of MDS, such as loss in 5q and 7q (8%, 3 of 37) and in 20q (5.5%, 2 of 37).

Loss also was frequently found in chromosomes 12, 17, and 18, specifically of the short arm of chromosomes 12 and 17 (21.5% and 19%, respectively) and the long arm of chromosome 18 (35%). The q arm of chromosome 11 was usually implicated in gain of material (35%).

Array CGH Analysis of Subtelomeric Regions

By HR-CGH, we repeatedly detected recurrent genomic loss and gain that involved chromosomal material within subtelomeric bands, such as deletions in 4p16, 5p15.3, 6q27, 8p23, 18p11.3, and 18q23, and gain in 1p36.3, 4p16, and 19p13.3 (Table 3 and Fig. 1). To validate these microalterations, we

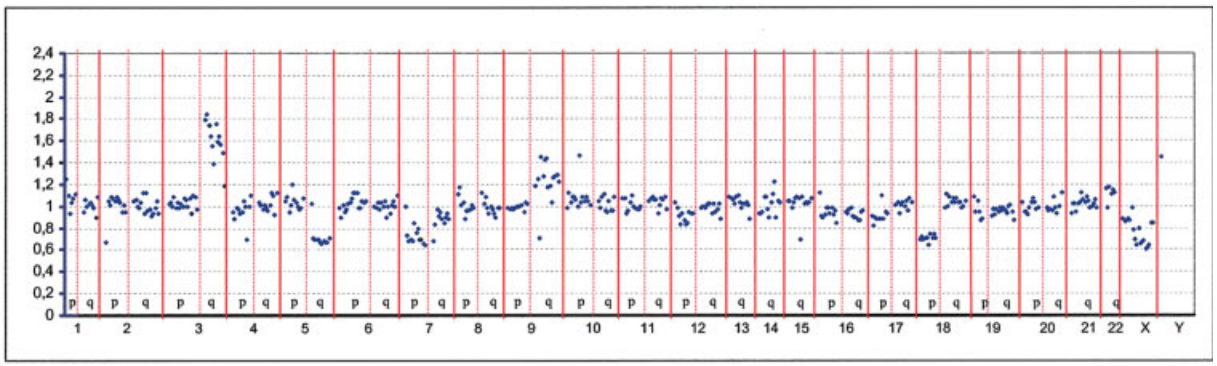

Figure 2. Results of subtelomeric array CGH from case 15. *Y* axis represents the normalized, averaged intensity ratio values for each clone in the array ordered by chromosome and position on the *X* axis. Amplification of 3qter and several clones of 9qter was detected. Gains affected subtelomeres 9qter and 22qter; losses affected 5qter, 7pter and 7qter, 12pter, and 18pter. The gain of 19pter detected by HR-CGH was not detected in this analysis.

performed array CGH analysis on cases 3, 15, and 22. These three cases showed eight copy number changes involving seven subtelomeric regions: −6q27, −18p11.3, and +19p13.3, in case 3; +19p13.3, in case 15; and −4p16, −5p15.3, −6q27, and −18q23, in case 22 (Table 2). The array CGH was constructed with BAC clones covering the first 5 Mb of each subtelomeric region (see Materials and Methods section for details). From this analysis, we first confirmed the genomic loss of 18p in case 3 but failed to see the remaining subtelomeric changes that were detected by HR-CGH in the other chromosomes and cases. Second, the subtelomeric array CGH showed the same large deletions and amplified regions in chromosome arms 3q, 5q, 7p, 7q, 8q, 9q, 12p, 18p, and 22q (Table 2 and Fig. 1), that had been seen with conventional CGH. In addition, subtelomeric alterations of varied size, both gains and losses, that had been missed with HR-CGH were detected with the CGH array (Fig. 2).

Regions of High-Level Gain (Amplification)

HR-CGH was able to identify amplifications of 20 regions in 13 patients (Fig. 1). 6 of whom showed two or three amplifications. The eight autosomal chromosomes in which these amplified regions were found were chromosomes 3, 8, 11, 17, 18, 20, 21, and 22. High-level gains affecting the sex chromosomes were found in three cases, in two of whom whole chromosome Y was involved (Table 3, Fig. 1).

Four patients had been analyzed previously by FISH because SKY had shown that they had extra chromosomal material involving chromosome bands 17q12, 11q23, and 21q21; in these cases, we confirmed amplification of the *ERBB2* (case 1), *MLL* (case 8), and *RUNX1* (case 9) genes (Martínez-Ramírez et al., 2004). In the present study, by using FISH, we were able to analyze another seven patients in whom high-level gains had been detected on HR-CGH (Fig. 3). In case 36, we observed extra material in 11q23. For this reason, we used the *MLL* gene probe to study its involvement in the amplification and were able to confirm *MLL* gene amplification in this case. Cases 25, 29, and 35, for whom CGH had shown large gains in 21q22, were analyzed with a *RUNX1* gene probe, which showed amplification of this gene in all patients. Cases 25 and 31 showed amplification in 18q12q21, which was studied by use of a *BCL2* gene probe. In case 25, the result was normal (2 signals), but in case 31, we did detect amplification of *BCL2*. Cases 15 and 35 also showed high-level gains in 3q27q29 (detectable in the subtelomere by array CGH; see Fig. 2) and 3q26q27, respectively. We used a FISH probe of the *BCL6* gene to study both cases. We found the *BCL6* gene to be amplified in case 15 and normal in case 35. Finally, in case 31, with additional material in chromosome region 8q23q24, FISH analysis with a *MYC*-specific probe showed only three copies of the gene, suggesting that gain of this region, detected by HR-CGH, could be produced by another gene and or genes.

DISCUSSION

Detailed information surrounding chromosomal abnormalities in hematologic malignancies is important because specific aberrations have strong prognostic value (Dohner et al., 1997; Greenberg et al., 1997; Grimwade et al., 1998). Furthermore, precise analysis of certain chromosomal rearrangements is a crucial step in the identification of genes

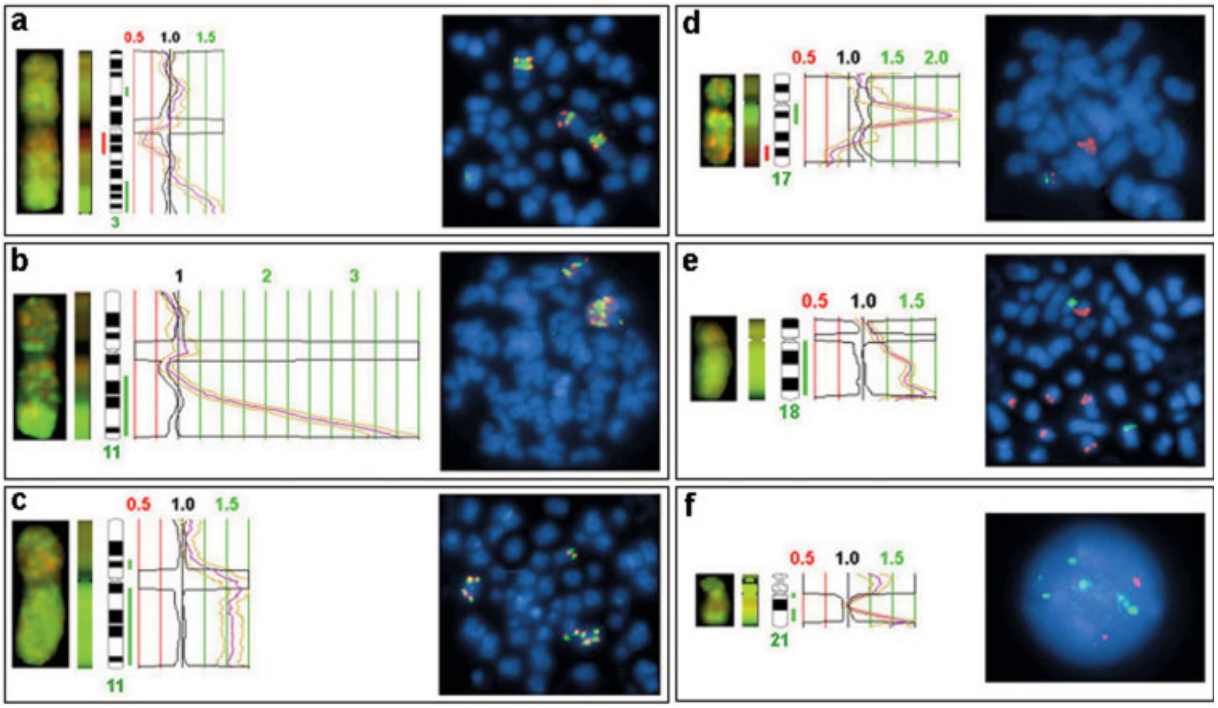

Figure 3. Amplification detected by CGH and confirmed by FISH: ratio profiles of chromosomes 3, 11, 17, 18, and 21 showing high-level gains to the right of the image and FISH showing genes involved in the amplifications; CGH profiles of chromosomes 3, 11, 17, 18, and 21 with amplifications in different regions and FISH images of different gene probes showing amplifications of the genes studied. (a) Case 15 with amplification in 3q27qter involving the *BCL6* gene (in 3q27). (b) Case 8 with amplification in 11q14qter involving the *MLL* gene (in 11q23). (c) Case 36 with amplification in 11q involving the *MLL* gene (in 11q23). (d) Case 1 with amplification in 17q12q21 involving the *ERBB2* gene (in 17q21). (e) Case 31 with amplification in 18q12q21 and 18q22 involving the *BCL2* gene (in 18q21). (f) Case 29 with amplification in 21q22 involving the *RUNX1* gene (in 21q22).

or chromosomes related to the pathogenesis of a disease and are important in patient prognosis. CKs are a major cause of incomplete G-banding analysis, and for this reason, molecular cytogenetics has been considered a powerful tool that complements conventional cytogenetics in these types of cases. In the present work, we analyzed 37 patients who had myelodysplastic syndromes with complex karyotypes, some of them previously defined by SKY techniques (Martínez-Ramírez et al., 2004), by using HR-CGH. The data obtained revealed a new picture of MDS with CK that included a better description of the structural and numerical alterations classically described, the definition of new genetic alterations, and the finding of microalterations detected only by CGH array.

By G-banding, we detected numerical and structural alterations similar to those described previously (Sandberg, 1990; Heim and Mitelman, 1995): monosomies 5, 7, 17, and 18, trisomy 8, and structural alterations affecting 5q, 11q, 17p, and 20q (Table 4). These alterations were redefined by CGH, and the total number of aberrations increased. For example, the percentage of cases with monosomies detected by HR-CGH and SKY was lower than that found by G-banding, whereas the number of structural aberrations detected increased, especially for the smaller chromosomes, such as chromosomes 17, 18, and 20 (Table 4). In addition, we found the 11q+ alteration to be one of the most frequently found by HR-CGH (38%), versus 13% and 0% by SKY and G-banding, respectively. These observations have been made in other studies on MDS or myeloid leukemia and CK that used molecular cytogenetic techniques (Kakazu et al., 1999; Lindvall et al., 2001; Mrozek et al., 2002; Schoch et al., 2002; Van Limbergen et al., 2002; Cigudosa et al., 2003) and could reflect the complexity of these alterations. Chromosomes missing by G-banding would really be rearranged internally, giving markers or derivative chromosomes that could be identified only by SKY and/or by CGH as interstitial deletions. On the other hand, the same can occur with other alterations detected by CGH such as the double gains or deletions of whole chromosomes (cases 1, 2, 10, and 21), which should be confirmed by complementary techniques because the CGH results could be overestimated.

TABLE 4. Comparison of Characteristic Alterations in MDS by Conventional Cytogenetics, HR-CGH and SKY

Chr.	CC (n = 29*)		CGH (n = 37)		SKY (n = 23)	
−5	$n = 4$	(14%)	$n = 1$	(2.7%)	$n = 1$	(4%)
−7	$n = 7$	(24%)	$n = 2$	(5.5%)	$n = 2$	(8.5%)
−17	$n = 2$	(7%)	$n = 0$	(0%)	$n = 1$	(4%)
−18	$n = 8$	(27.5%)	$n = 2$	(5.5%)	$n = 4$	(17%)
−20	$n = 5$	(17%)	$n = 0$	(0%)	$n = 2$	(8.5%)
+8	$n = 5$	(17%)	$n = 7$	(19%)	$n = 6$	(26%)
3q−	$n = 3$	(10%)	$n = 9$	(24%)	$n = 5$	(22%)
5q−	$n = 14$	(48%)	$n = 26$	(70%)	$n = 12$	(52%)
7q−	$n = 1$	(3.5%)	$n = 12$	(32.5%)	$n = 5$	(22%)
11q−	$n = 3$	(10%)	$n = 10$	(27%)	$n = 5$	(22%)
11q+	$n = 0$	(0%)	$n = 14$	(38%)	$n = 3$	(13%)
12p−	$n = 2$	(7%)	$n = 8$	(21.5%)	$n = 3$	(13%)
17p−	$n = 1$	(3.5%)	$n = 7$	(19%)	$n = 6$	(26%)
18q−	$n = 2$	(7%)	$n = 14$	(38%)	$n = 4$	(17%)
20q−	$n = 3$	(10%)	$n = 9$	(24%)	$n = 5$	(22%)

Chr: chromosome; CC: conventional cytogenetics
*In 8 patients, we only know the chromosome number and that a complex karyotype was presented.
HR-CGH: high-resolution comparative genomic hybridization; SKY: spectral karyotyping (data from Martinez-Ramirez et al., 2004).

Although genomic amplification occurs mainly in solid tumors, we found regions of high-level amplification in 35% of patients (13 of 37). They were detected in different autosomal chromosomes, some of them previously identified, such as 3q26–27, 11q23, 17q12, 18q12–21, and 21q21, and in both sex chromosomes. In previous work, we used FISH to confirm amplification of the *ERBB2* (17p12), *MLL* (11q23), and *RUNX1* (22q21) genes in four patients (cases 1, 8, 9, and 23; Martínez-Ramírez et al., 2004). We extended our study to another seven patients by using the same probes and three new ones (*MYC*, *BCL2*, and *BCL6*), also located in the rest of the chromosomes with genomic gains. We found *MLL* gene amplification (>15 copies) in patient 36, confirming that amplification of the *MLL* gene is a recurrent genetic alteration in myeloid neoplasias (Cuthbert et al., 2000; Michaux et al., 2000; Streubel et al., 2000; Lindvall et al., 2001; Cigudosa et al., 2003). Recently, Pope et al. (2004) demonstrated that in a series of myeloid malignancies with 11q23 gain, this amplicon is invariably accompanied by *MLL* amplification and overexpression. In addition, they demonstrated there was significant up-regulation of *MLL* in MDS in an unselected series of MDS and AML samples. These data confirm *MLL* as an important target of the 11q23 amplicon and provide further evidence of an etiologic role for *MLL* gain of function in this malignancy.

Another region with recurrent high-level gains was 21q22, which was found in three patients (cases 25, 29, and 35), in all of whom we were able to identify amplification of the *RUNX1* gene (Fig. 3). An increase in copy number of the *RUNX1* gene has been reported in AML patients, some of them secondary to MDS (Kakazu et al., 1999; Streubel et al., 2000; Hilgenfeld et al., 2001), but our cases were MDS at diagnosis. Therefore, *RUNX1* amplification can also be a frequent alteration in this type of patient. In two other cases (cases 25 and 31), we detected amplifications by HR-CGH in 18q12–q21. Using FISH, we analyzed the *BCL2* gene, which is in 18q21. Case 31 presented a high number of copies of the *BCL2* gene in different chromosomes, whereas the other case was normal. HR-CGH also showed amplification of 3q27 in two patients. Given that the *BCL6* gene is in this region, we performed a FISH analysis with the *BCL6* probe, finding high-level gain (>10 copies) in case 15 but case 35 to be normal. *BCL2* and *BCL6* are genes related to apoptosis, and their deregulation may result in perturbation of the proteins that control apoptosis, leading to abnormally enhanced growth and expansion of leukemic cells. The apoptosis could be induced or inhibited by these genes, depending on the level of expression and the cellular context, as has been observed in non-Hodgkin lymphoma, where both genes are usually rearranged by translocations, yielding high-level expression (Ye et al., 1995; Aster and Longtine, 2002; Baron et al., 2002; Fernandez et al., 2002). As far as we know, this is the first report of amplifications involving genes related to apoptosis in MDS with CK and could represent another mechanism leading to genetic overexpression. Further studies involving other groups of patients and levels of expression of the proteins encoded by these genes will be necessary to evalu-

ate their possible pathogenetic role in myeloid malignancies.

HR-CGH analysis of different chromosomes showed alterations in subtelomeric regions, including deletions in 4p16, 5p15.3, 6q27, 8p23, 18p11.3, and 18q23 and gains in 1p36.3, 4p16, and 19p13.3 (Table 3 and Fig. 1). Because HR-CGH includes analysis of chromosome regions normally excluded from conventional CGH analysis, such as subtelomeric regions, we wanted to confirm these changes with an alternative method. We took advantage of the existence of an array CGH platform dedicated to the subtelomeric regions of the human genome. The array CGH analysis confirmed only one alteration in the three cases we studied. However, we found other alterations affecting only a BAC or a small number of BACs that HR-CGH was unable to detect. This result suggests that HR-CGH may overestimate alterations involving subtelomeric bands because of software problems in these regions and may miss the existence of others whose deletions or gains are small. Thus, these regions continue to be problematic despite the improvement that the HR-CGH has achieved, and they should be discarded in CGH studies.

In summary, a combination of conventional and molecular cytogenetic techniques has provided us with a more precise characterization of this type of MDS with complex karyotypes. (a) Monosomies 5, 7, 18, and 20, which had been detected by G-banding, were reclassified by HR-CGH as frequent structural rearrangements of the same chromosomes. In addition, we identified a new 11q+ structural alteration, which we found in nearly 40% of the cases in this study. (b) About 35% of cases analyzed by HR-CGH showed a high level of gains produced by amplifications of genes such as *MLL*, *RUNX1* and *ERBB2*, previously described in myeloid neoplasias, and new genes such as *BCL2* and *BCL6* that are related to apoptosis. (c) Finally, we do not believe that telomeric alterations detected by HR-CGH can be true alterations, but array CGH suggests that small deletions in subtelomeric regions can be frequent in patients who have MDS with CK. This type of alteration could be extended to other neoplasias and could play an important role in the stability of chromosomes related to the development and progression of the disease. However, further studies are necessary for an understanding of the meaning of these changes.

ACKNOWLEDGMENTS

We thank the COST program, Action B19, Molecular Cytogenetics of Solid Tumours, for funding an STSM to David Blesa at the Department of Medical Genetics, University Hospital Groningen. We thank Blanca Fernández Martínez, M[a] Carmen Martín Guijarro, and Amanda Wren for their technical assistance.

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The Accumulation of Specific Amplifications Characterizes Two Different Genomic Pathways of Evolution of Familial Breast Tumors

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Abstract **Purpose and Methods:** High-level DNA amplifications are recurrently found in breast cancer, and some of them are associated with poor patient prognosis. To determine their frequency and co-occurrence in familial breast cancer, we have analyzed 80 tumors previously characterized for *BRCA1* and *BRCA2* germ-line mutations (26 *BRCA1*, 18 *BRCA2*, and 36 non-*BRCA1/2*) using high-resolution comparative genomic hybridization.

Results: Twenty-one regions were identified as recurrently amplified, such as 8q21-23 (26.25%), 17q22-25 (13.75%), 13q21-31 (12.50%), and 8q24 (11.25%), many of which were altered in each familial breast cancer group. These amplifications defined an amplifier phenotype that is correlated with a higher genomic instability. Based on these amplifications, two different genomic pathways have been established in association with 8q21-23 and/or 17q22-25 and with 13q21-31 amplification. These pathways are associated with specific genomic regions of amplification, carry specific immunohistochemical characteristics coincident with high and low aggressiveness, and have a trend to be associated with *BRCA1* and *BRCA2/X*, respectively.

Conclusion: In summary, our data suggest the existence of two different patterns of evolution, probably common to familial and sporadic breast tumors.

Germ-line mutations of *BRCA1* (OMIM 113705) or *BRCA2* (OMIM 600185) confer an estimated cumulative lifetime risk of 56% to 84% of breast cancer and a 15% to 45% risk of ovarian cancer (1, 2). High-risk families are usually selected based on specific clinical criteria to perform a genomic screening of these genes, but their mutations only explain 30% of familial breast cancers (3). Several candidate loci such as 8p12-p22 (4), 13q21 (5), and 2q32 (6) have been postulated to represent the genetic background of the remaining 70% of families (group named BRCAX) without definitive results.

Immunohistochemical and expression profiles have been defined for familial breast cancers associated with distinct classes of *BRCA* tumors. Briefly, *BRCA1* tumors are high grade, negative for hormone receptors and HER-2, positive for p53, E2F6, cyclins A, B, and E, SKP2, and topoisomerase IIα and display a high expression of P-cadherin (7–10). In contrast, *BRCAX* tumors have a lower grade and an opposite phenotype which is hormone receptor and HER-2 positive, a low proliferation rate, and undetectable P-cadherin expression (9, 10). *BRCA2* tumors show an intermediate phenotype with a higher proliferation rate than *BRCAX*, and positive markers different from *BRCA1* such as cyclins D1 and D3, p27, p16, p21, and cyclin-dependent kinases 4, 2, and 1 (7–10).

Hereditary breast tumors have also been studied characterizing their genomic changes using comparative genomic hybridization (CGH; ref. 11). Some of the genomic changes present in these tumors have been used to build classifiers that distinguish between sporadic and *BRCA1/2* tumors (12, 13) or between *BRCAX* and *BRCA1/BRCA2* tumors (14). This last predictor, which uses high-resolution CGH (HR-CGH), allowed us to identify *BRCAX* cases with a genomic changes profile similar to *BRCA1* tumors, probably due to aberrant methylation of the *BRCA1* promoter (14).

These studies have also shown the existence of common alterations such as high-level DNA amplifications that, in fact, are recurrently found in familial and sporadic breast cancer (15). Some of these amplified regions include known oncogenes, such as *MYC* (8q24), *ERBB2* (17q12), *FLG* (8p12), *CCND1* (11q13), and *IGFR-I/FES* (15q24-q25; refs. 16, 17); some of these oncogenes, such as *MYC* and *ERBB2*, have largely been correlated with poor prognosis (18, 19). However, in other cases, specific high-level amplifications, such as 8q23, 17q23-q25, and 20q11-q13, have been observed at chromosomal sites that do not coincide with the locations of the classic

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Received 6/13/05; revised 9/8/05; accepted 9/30/05.

Grant support: Ministerio de Ciencia y Tecnología grant SAF03-02497 and Red Temática de Investigación Corporativa Centros de Cáncer grant C0-010.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-05-1278

breast cancer oncogenes (20). The comprehensive characterization of some of these amplicons has already started (21, 22) but their meaning remains unclear, especially because some studies have shown that there are tumors with a trend to accumulate high-level DNA amplifications (23–25).

In the present study, we have used HR-CGH to estimate the frequency and distribution of regions with high-level amplifications in familial breast tumors because this genomic event may have important prognostic value. We have classified the cases according to the number of amplifications and defined their genomic characteristics and the immunohistochemical profiles associated with specific amplification pathways.

Materials and Methods

Patients and tumor samples. Eighty breast tumors were collected from patients selected from three centers in Spain: the Spanish National Cancer Centre (CNIO), the Fundación Jimenez Díaz in Madrid, and the Hospital Sant Pau in Barcelona. Patients belonged to families with at least three women affected with breast/ovarian cancer and at least one of them diagnosed before 50 years, or to families with female breast/ovarian cancer and at least one case of male breast cancer. All cases had been studied for mutations in the *BRCA1* and *BRCA2* genes using standard procedures (26). Twenty-six cases presented mutations in the *BRCA1* gene, 18 cases had mutations in the *BRCA2* gene, and 36 cases were negative for germ-line mutations in these genes and were considered as *BRCAX*.

High-resolution comparative genomic hybridization analysis. Genomic DNA was isolated from 4 × 10-μm sections of 80 paraffin-embedded tumors using a commercially available DNase Tissue Kit (Qiagen, Chatsworth, CA) according to the recommendations of the manufacturer. HR-CGH was carried out as described in our recent study that included 72 of these cases, and for genomic studies, we used the 63 regions previously defined (14). Briefly, we chose as the most common minimal regions of involvement 50 regions including imbalances in at least 30% of the *BRCA* cases used to build our previous predictor (14) and with at least three cases defining the cytogenetic thresholds. To include the rest of the genome not fitting the previously defined criteria, we grouped the unselected areas on 13 chromosomal regions. Those chromosomal regions with CGH ratios >1.5 were defined as high-level amplifications and considered as recurrent when they were found in two or more cases.

Tissue microarray and immunohistochemistry. For immunohistochemistry studies, we used a previously published tissue microarray (8, 9) that included 74 of the breast tumors here analyzed by HR-CGH; 23 were *BRCA1*, 18 were *BRCA2*, and 33 were *BRCAX*.

Before tissue microarray construction, total sections of each H&E-stained tumor were evaluated and classified according to the WHO classification. Grade was assessed using the Nottingham grading system.

Immunohistochemical staining was done by the Labeled Streptavidin Biotin method (DAKO, Glostrup, Denmark) with a heat-induced antigen retrieval step. Sections from the tissue array were immersed in boiling 10 mmol/L sodium citrate at pH 6.5 for 2 minutes in a pressure cooker. Antibodies, dilutions, and suppliers are listed in Supplementary Table S1. Two pathologists (E.H. and J.P.) independently evaluated in a blind study the immunohistochemical staining of nine antibodies. The percentage of stained nuclei, independent of the intensity, was scored for estrogen receptor, progesterone receptor, p53, and Ki-67. In the same way, the percentage of cells with cytoplasmic stain was scored for Bcl-2.

We took the mean of the percentage of stained cells as the cutoff point. Thus, when the percentage of stained cells was ≥10%, we considered the tumor as positive for estrogen receptor and progesterone receptor; ≥25%, positive for p53; and ≥70%, positive for Bcl-2. Three categories were defined for Ki-67: 0% to 5%, 6% to 25%, and >25% of stained nuclei.

A tumor was considered to have preserved expression of E-cadherin and catenins (γ-catenin and $p120^{ctn}$) when >75% of the cells showed complete membranous staining of similar intensity as normal breast epithelium (27).

Statistical analysis. We used a nonparametric Mann-Whitney *U* test to identify differences in the number of genomic changes (chromosomal gains or losses) among the patient groups (based on the BRCA class or the number of high-level amplifications). Differences in the frequency of involvement of individual chromosomal regions among the three familial breast cancer classes were tested with Fisher's exact test. The indicated *P* values were calculated using the Stat POMELO (28). This tool is available at http://pomelo.bioinfo.cnio.es. To determine immunohistochemical differences between groups based on the main amplified region, the χ^2 contingency test was used with Fisher's exact test correction when necessary. The SPSS for Windows statistical program (SPSS, Inc., Chicago, IL) was used for this analysis. Hierarchical unsupervised clustering was done using the UPGMA method (28). The statistical test and the clustering are implemented in the GEPAS package (http://bioinfo.cnio.es).

Results

In the present study analyzing 80 tumors by HR-CGH, we have found similar results with our previous work that included 72 of them (14). Briefly, the mean number of changes was higher in *BRCA1* and *BRCA2* than in *BRCAX* tumors. Four chromosomal regions were commonly altered in ~50% of the three *BRCA* groups: gains at 1q and 8q21-23 and losses at 8p21-23 and 11q22-25. There were also specific changes that significantly distinguished each *BRCA* class in the intergroup comparisons ($P < 0.05$, nonadjusted Fisher's exact test). The overall frequencies of changes in the 63 regions are shown in Supplementary Table S2.

Identification of recurrent regions with high-level DNA amplification

We found 21 chromosomal regions recurrently amplified (HR-CGH ratio > 1.5, present in two or more cases). The most frequently involved cytogenetic regions were 8q21-23 (21 of the 80 cases studied), 17q22-25 (11 cases), 13q21-31 (10 cases), 8q24 (9 cases), 12q15-21 (7 cases), and 20q12-13, 15q22-26, and 3q25-26.3 (6 cases each; Fig. 1). The most frequently altered regions were common to all the *BRCA* groups except 17q22-25 and 20q12-13, which were not present in the *BRCA2* mutation carriers.

No amplified regions were found in ~50% of cases of the three groups (39 cases); these cases were named "non-amplifier" (NA) tumors, in contrast to the rest of cases that had at least one amplified region. Based on the median number of amplifications, we established a cutoff that distinguished two further categories: "low-amplifier" (LA) tumors that showed one or two regions with high-level DNA amplifications (lower than the median number; 18 cases) and "high-amplifier" (HA) tumors which had three or more amplified regions (equal to or more than the median number; 23 cases). The distribution of these categories within the distinct *BRCA* classes is shown in Fig. 2. *BRCA1* tumors seem to have a higher trend to accumulate amplifications than *BRCA2* or *BRCAX* tumors although the three groups do not present significant differences. As expected, those tumors with more amplified regions had significantly more genomic alterations. HA tumors have a higher genomic instability than the other phenotypes, and the same occurs with LA tumors

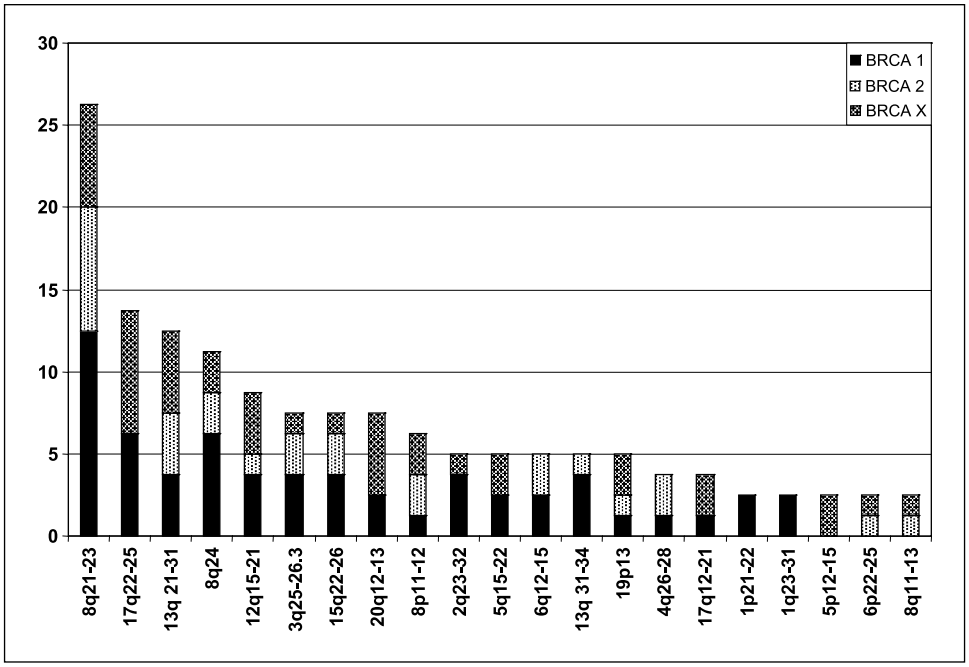

Fig. 1. Distribution of the 21 chromosomal regions recurrently found amplified among the 80 familial breast cancer samples studied.

that present higher instability than NA tumors (Supplementary Fig. S1).

Two different pathways of genomic evolution

Association with specific amplifications. We have analyzed the association of the amplified regions with each other using an unsupervised clustering (Fig. 3A). Two main branches were separated, one of them associated with 13q21-31 amplification which presented a trend to accumulate specific genomic amplifications such as 6q12-15, 5q15-22, 2q23-32, or 4q26-28, and a second one associated with amp8q21-23 and/or 17q22-25, with 15q22-26 or 8q24 regions in the amp8q21-23 group and the 20q12-13 region in the amp17q22-25 tumors. When comparing the frequencies of amplification of the regions between both branches, we observed significant differences (adjusted $P < 0.05$) for 13q21-31 ($P = 0.000$), 8q21-23 ($P = 0.0017$), and 6q12-15 ($P = 0.017$; data not shown). Other genomic changes (gains and losses) were also distributed in a significantly different fashion between both branches (data not shown). When analyzing the distribution of the three BRCA groups, we observed that they were randomly distributed among the two main branches.

Association with immunohistochemical markers. To determine if there were specific characteristics associated with the acquisition of the amplified regions, we defined the immunohistochemical profile by analyzing histologic variables and some markers of proliferation (Ki-67), hormone receptors (estrogen receptor and progesterone receptor), the cell cycle (p53), cell adhesion ($p120^{ctn}$, CAD-E, CAD-P, and G-CAT), and apoptosis (Bcl-2). Statistical comparisons were made to identify the markers that were significantly different in these groups (Table 1). Tumors with amp8q21-23 and/or 17q22-25 were characterized by a higher grade and mitosis number, a high expression of Ki-67, and negative staining for $p120^{ctn}$ and E-cadherin. By contrast, cases with amp13q21-31 mainly presented a low grade and mitosis, a low level of Ki-67, positive staining for hormone receptors (estrogen receptor and progesterone receptor) in nearly 100% of the cases, negative

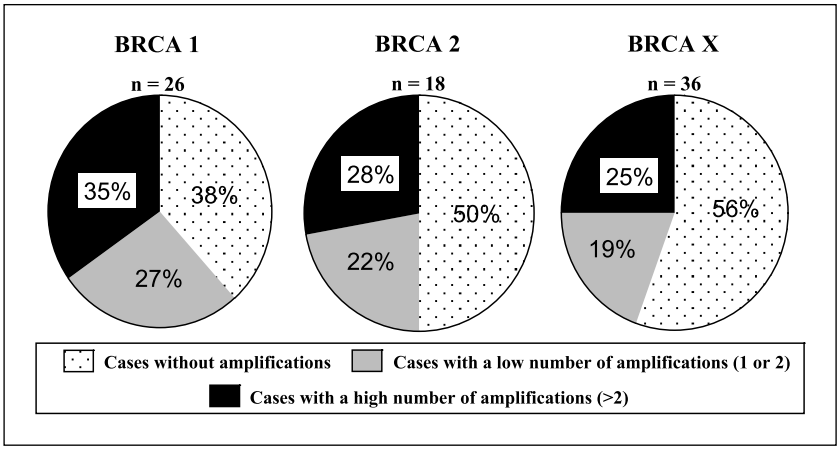

Fig. 2. Distribution of the three genomic phenotypes (HA, LA, and NA tumors) described in this study within the different *BRCA* classes.

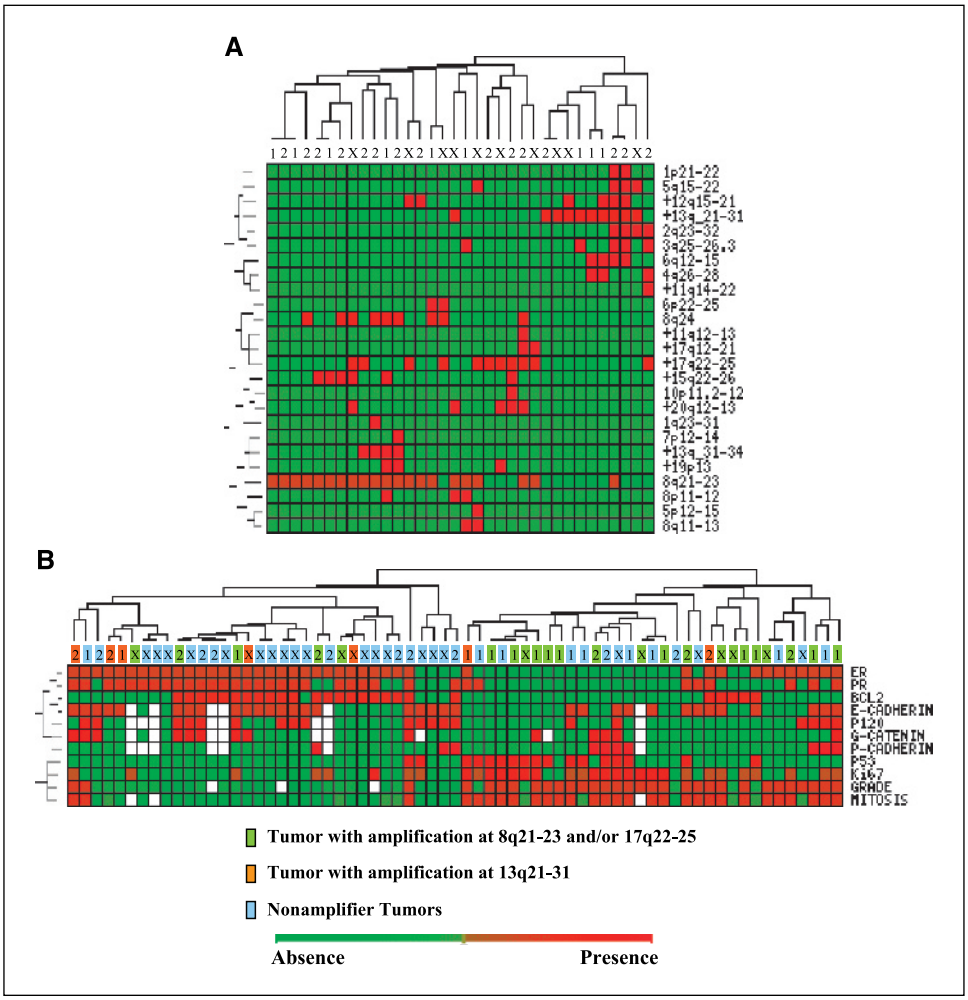

Fig. 3. *A*, unsupervised cluster with the 25 amplified regions (21 recurrent and 4 nonrecurrent) and 34 cases that had at least one amplification at 8q21-23, 13q21-31, or 17q22-25. Red, presence of amplification; green, absence of amplification. Two main groups are represented. The first one with amplification at 13q21-31 and other secondary amplifications (*right*), and the second branch with amplification at 8q21-23, with or without amplification at 17q22-25 and other secondary amplifications (*left*). *B*, unsupervised cluster with immunohistochemical markers in 66 studied cases. Amplified cases without amp8q21-23, 13q21-31, or 17q22-25 were not taken into account. Red, positive expression; green, negative expression; the intensity of the color is a function of the immunohistochemical expression level. White, nonevaluable expression. The right branch presents markers associated with aggressiveness, proliferation, and amplification at 8q21-23; the left branch includes characteristics of low aggressiveness, good prognosis, amplification at 13q21-31, and nonamplifier tumors.

staining for p53 and $p120^{ctn}$, and positive staining for E-cadherin; this latter marker was significantly different from the NA tumors. In addition, NA tumors presented an immunohistochemical phenotype similar to amp13q21-31 tumors although with a lower percentage of cases positive for hormone receptors and negative for $p120^{ctn}$ (Table 1).

Using the studied immunohistochemical variables, we made with all cases an unsupervised cluster to determine if these tumors are well separated (Fig. 3B). The cluster had two main branches: one was associated with high grade and mitosis, a high level of Ki-67, negativity for hormone receptors and Bcl-2, and positive for p53 (right); the other branch showed an opposite phenotype with lower grade and mitosis, a lower level of Ki-67, positivity for hormone receptors and Bcl-2, and negative for p53 (left). The first branch contained the NA *BRCA1* tumors and the majority of cases with amp8q21-23 and/or 17q22-25 (18 cases) independent of the *BRCA* type. In the left branch, there was a mix of NA *BRCA2/X* tumors (23 cases), amp13q21-31 (5 cases), and amp8q21-23 (5 cases).

Discussion

In the present study, we have established the most frequently found somatic alterations in familial breast tumors by analyzing 63 chromosomal regions by HR-CGH. We have defined the existence of a genomic phenotype that is characterized by the accumulation of DNA amplifications that are commonly found in the three *BRCA* groups. Finally, we have shown three major amplified regions that define two different genomic pathways that seem to be associated with specific immunohistochemical characteristics and prognosis.

We have found four common genomic alterations present in the three classes of *BRCA* tumors: gains at 1q and 8q21-23 and losses at 8p21-23 and 11q22-25. These alterations coincide with those described in other studies on *BRCA* mutation carriers (11, 13) and with our previous study, although the gain at 8q21-23 had lower frequency in *BRCAX* cases (14). It is interesting to note that van Beers et al. (13) included 80 regions with alterations defined by CGH in a similar way as described in the present study, and the majority of these regions coincide with ours. Therefore, these altered regions could represent a core of abnormalities common to familial and sporadic breast cancer.

We also found specific regions associated with the three *BRCA* groups (Supplementary Table S2). However, only loss at 5q11-23 in *BRCA1* and gain at 3q11-23 in *BRCA2* coincide with the specific changes associated with some of the *BRCA* classes defined by van Beers et al. (13). These specific chromosomal regions warrant further analysis.

Finally, we have defined three different genomic phenotypes: NA, with no amplification (48% of the total number of

samples); LA, with one or two amplified regions (23%); and HA, with three or more amplifications (29%). As expected, tumors with high-level DNA amplifications presented higher instability than NA tumors. These results support the idea that tumors with a high genomic instability have an increased probability to develop multiple amplifications (18). In this scheme, LA tumors would represent the first level of genomic instability to develop more amplifications.

Different pathways of genomic evolution. Among the 21 regions with high-level DNA amplification that we have identified on familial breast cancer, three amplified regions characterize two different genomic pathways. 8q21-23 and 13q21-31 are the two regions with the highest frequency of amplifications in both LA and HA tumors and may represent the earliest amplified regions that drive the tumors through distinct pathways of evolution (Fig. 4). In contrast, amp17q22-25, the second most frequent amplification, may be associated with tumor progression because it seemed to be significantly more frequent in HA tumors and was often amplified together with 8q21-23 (Fig. 4). By using unsupervised clustering, we

Table 1. Comparison of immunohistochemical variables among familial breast tumors with amplification at 8q21-23 and/or 17q22-25, amplification at 13q21-31, and without amplifications

	Cases with amp8q21-23 and/or amp17q22-25	*P**	Cases with amp13q21-31	*P*†	Nonamplifier cases	*P*‡
	n (%)		*n* (%)		*n* (%)	
Grade						
1	2 (9.1)	NS	1 (11.1)	NS	13 (37.1)	0.028
2	6 (27.3)		4 (44.4)		9 (25.7)	
3	14 (63.6)		4 (44.4)		13 (37.1)	
Mitosis						
1	3 (15.0)	NS	4 (44.4)	NS	20 (55.6)	0.006
2	4 (20.0)		1 (11.1)		4 (11.1)	
3	13 (65.0)		4 (44.4)		12 (33.3)	
Ki-67						
0-5%	7 (30.4)	NS	5 (62.5)	NS	24 (64.9)	0.040
6-25%	9 (39.1)		2 (25.0)		7 (18.9)	
>25%	7 (30.4)		1 (12.5)		6 (16.2)	
Estrogen receptor						
Negative	11 (47.8)	0.028	0	NS (0.085)	14 (36.8)	NS
Positive	12 (52.2)		8 (100)		24 (63.2)	
Progesterone receptor						
Negative	14 (60.9)	0.037	1 (12.5)	NS (0.115)	18 (47.4)	NS
Positive	9 (39.1)		7 (87.5)		20 (52.6)	
p53						
Negative	16 (69.6)	NS	7 (87.5)	NS	28 (75.7)	NS
Positive	7 (30.4)		1 (12.5)		9 (24.3)	
$p120^{ctn}$						
Negative	15 (78.9)	NS	8 (100)	0.013	16 (50.0)	0.023
Positive	4 (21.1)		0		16 (50.0)	
E-Cadherin						
Negative	12 (60.0)	NS	3 (37.5)	NS	12 (36.4)	0.047
Positive	8 (40.0)		5 (62.5)		21 (63.6)	
P-Cadherin						
Negative	16 (80.0)	NS	8 (100)	NS	26 (81.3)	NS
Positive	4 (20.0)		0		6 (18.8)	
γ-Catenin						
Negative	13 (72.2)	NS	6 (75.0)	NS	25 (78.1)	NS
Positive	5 (27.8)		2 (25.0)		7 (21.9)	
Bcl-2						
Negative	14 (63.6)	NS	5 (62.5)	NS	22 (59.5)	NS
Positive	8 (36.4)		3 (37.5)		15 (40.5)	

NOTE: All *P* values were obtained by the χ^2 contingency test using Fisher's exact test correction when necessary. $P < 0.05$ was considered significant. NS, non-significant differences.

**P* obtained by comparison of cases with amp8q/17q versus amp13q.

†*P* obtained by comparison of cases with amp13q versus nonamplifier tumors.

‡*P* obtained by comparison of cases with amp8q/17q versus nonamplifier tumors.

could confirm these points: we observed that the branch represented by amp8q21-23 is associated with specific regions such as 15q22-26 and 8q24, and that the second branch defined by amp13q21-31 is associated with 6q12-15, 5q15-22, 2q23-32, and 4q26-28 (Fig. 3A). Therefore, the pathways defined by 13q21-31 and 8q21-23, with or without 17q22-25, might influence the future accumulation of other specific amplified regions and would determine the genomic evolution of these tumors. Genomic evolution is a frequent event in breast cancer (25, 29–32). In this way, Courjal and Theillet (29) and Courjal et al. (32) described the genetic evolution of a set of sporadic breast tumors based on amplified regions. Because the amplified regions they found affected the same chromosomes and in similar regions as in our genomic pathways, we think that 13q21-31, 8q21-23, and 17q22-25 may be common genomic amplification pathways in breast cancer.

Because of the genomic differences between the two pathways, we have tried to define the immunohistochemical characteristics associated with each one of them. We analyzed 11 immunohistochemical variables and found that tumors with amp13q21-31 presented a profile defined by positive estrogen receptor and progesterone receptor staining in 100% and 90% of cases, respectively, a low Ki-67 expression, and negative p53 and $p120^{ctn}$ staining (Table 1). The majority of these markers have been previously correlated with good prognosis and low malignant potential in *BRCA2* and *BRCAX* familial tumors (7–10), and although the number of tumors here analyzed is small, the majority correspond to these subtypes. In contrast, the group associated with amp8q21-23 with or without amp17q22-25 showed immunohistochemical variables of aggressiveness, such as a high grade and mitosis number, high expression of Ki-67, and negative expression of hormone receptors and E-cadherin. Moreover, recent studies by our group and others have shown that the immunohistochemical characteristics of *BRCA1* tumors are in general represented by this profile (7–10), and although in the amp8q21-31/17q22-25 group all three subtypes are represented, the majority are *BRCA1* tumors. Regarding NA tumors, the immunohistochemical profile was similar to that of the amp13q21-31 group.

The unsupervised cluster showed the same trend (Fig. 3B): the right branch included phenotypic markers of aggressiveness and mainly contained NA *BRCA1* tumors (8 cases) and *BRCA1* and *BRCA2/X* tumors with amp8q21-31/17q22-25 (10 and 8 cases, respectively); in the left branch, we found NA *BRCA2/X* tumors (23 cases) and *BRCA1* and *BRCA2/X* tumors with amp13q21-31 (1 and 4 cases, respectively). Only a minority of cases was incorrectly located according to the amplified region or *BRCA* type. All these data suggest that the genetic mutation in familial breast tumors is mainly responsible for the immunohistochemical phenotype, although a small group can present different phenotypes (more or less aggressive) probably due to their own genetic background. During tumor evolution, the genetic phenotype may induce a set of genomic changes through two main pathways: amp13q21-31, which is associated with less aggressive tumors and good prognosis, and amplification of the 8q21-23/17q22-25 region, which is associated with highly aggressive tumors and bad prognosis. Thus, *BRCAX* cases that amplify 8q21-23 and/or 17q22-25 may represent a more aggressive subgroup within the heterogeneous population of *BRCAX* tumors, and this should be taken into account in future searches for genes responsible for BRCAX.

Why this occurs is currently unknown but different studies have pointed out that the gain of 8q is a recurrent event in sporadic breast cancer with poor prognosis (23, 24, 33, 34). Our results support this correlation and suggest that 8q amplification may be a universal bad prognostic marker for breast cancer.

The same occurs with the amplification 17q22-25, which is distal to *ERBB2* and *BRCA1* genes and seems to be a major amplification site in sporadic breast cancer (20, 29, 35–37). This amplified region has also been correlated with poor patient prognosis (24, 33) and detailed characterizations of

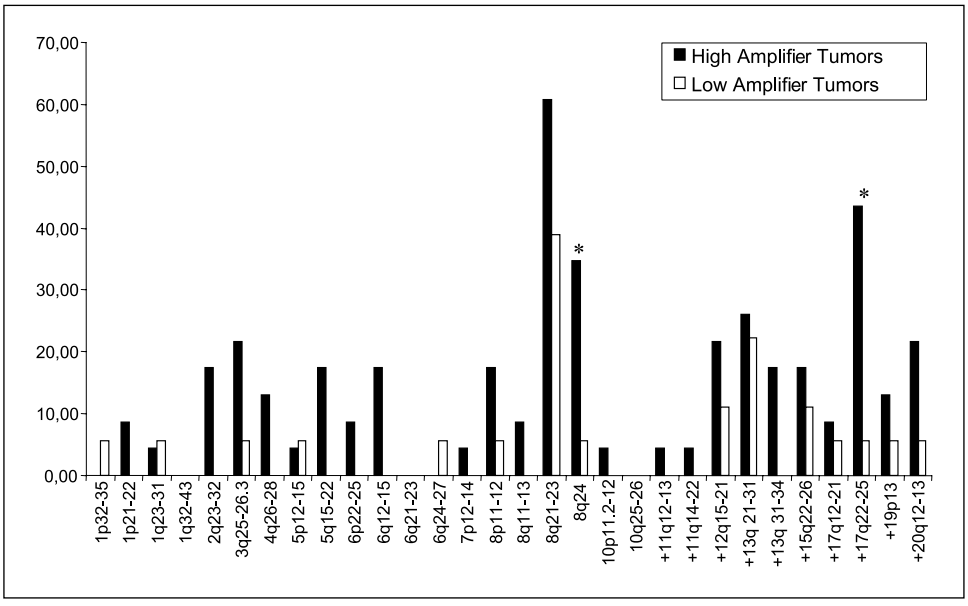

Fig. 4. Frequency of the 21 recurrent amplified regions found in the HA (*black columns*) and LA tumors (*white columns*). Amplifications at 8q21-23 and 13q21-31 are present with a high frequency in both groups. However, amplifications at 8q24 and 17q22-25 occur more frequently on HA tumors and may be considered as late alterations in the tumor development. *, significant differences in the statistical comparison of the frequencies between HA and LA tumors.

the amplification and overexpression of genes located at this region have already been reported (21, 38–41).

Finally, the amp13q21-31 group and its correlation with good prognosis still have to be studied in detail. Different candidate genes are located in this region, such as protocadherins (*PCDH9* and *PCDH17*), mitotic control protein genes (*KIAA1008*), Kruppel-like transcription factors (*KLF5* and *KLF12*), transcription regulatory function genes (*LMO7*), or inhibitors of natural killer activity and prostaglandin synthesis during pregnancy (*PIBF1*; refs. 5, 42, 43). 13q21-31 amplification has also been reported in breast cancer cell lines (20, 44, 45) but little is yet known on its role. Our findings about the recurrence of this amplification in all *BRCA* groups, but mainly in *BRCA2* and *BRCAX*, the strong correlation with estrogen receptor–positive tumors (100%), and the good prognosis immunohistochemical profile indicate that this amplified region could serve as a marker for tumor evolution and follow-up of the patient.

In summary, we have defined 21 recurrent amplification sites and described an amplifier phenotype that is probably common not only to familial but also to sporadic breast tumors and that is correlated with a higher genomic instability. In addition, two different genomic pathways associated with 8q/17q and 13q amplification have been established. They are associated with specific genomic regions of amplification, carry specific immunohistochemical characteristics, and have a trend to be associated with *BRCA1* and *BRCA2/X*, respectively. According to these data, tumors with 13q amplifications are associated with low aggressiveness and good prognosis whereas 8q/17q amplification defines tumors with high aggressiveness and poor prognosis. Because these amplifications have been previously identified in sporadic breast tumors and correlated with prognosis, the two pathways may represent a general mechanism of evolution in breast tumors and may contain key genes for tumor evolution.

Acknowledgments

We thank the Spanish National Tumor Bank Network, the Immunohistological Unit of the Spanish National Cancer Centre, and Carmen Martin from the Cytogenetics Unit for their technical support; Roger Milne and Ramón Díaz-Uriarte for their assistance in the statistical analysis; and Miguel Urioste, Laura Valle, and Raquel Rodríguez for the selection of families.

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European Journal of Human Genetics (2005), 1–9

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ARTICLE

About the origin and development of hereditary conventional renal cell carcinoma in a four-generation t(3;8)(p14.1;q24.23) family

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Conventional renal cell carcinoma (CRCC) may appear in families with germline translocations involving chromosome 3, although a recurrent responsible gene has not been found. We recently described a family with CRCC and a constitutional t(3;8)(p14.1;q24.23), and we demonstrated that no genes were disrupted by the translocation breakpoints. In order to investigate the genetic origin and features of the CRCC tumors that occurred in this family, we have extended the pedigree up to four generations, and analyzed peripheral blood samples from 36 members, CRCC tumors, normal renal tissues, and a gastric tumor. (1) By means of comparative genomic hybridization (CGH), we have detected loss of the derivative chromosome carrying 3p in all CRCC but not in the corresponding normal renal tissue. In addition, by means of the fluorescence *in situ* hybridization technique, we have observed that not all tumoral cells lose the der(3p), which suggests that, previous to this loss, another hit should occur to initiate the transformation of normal into tumoral cells. (2) All known mechanisms of inactivation of the candidate von Hippel-Lindau (VHL) gene have been studied in the tumors, detecting alterations in 65% of them. This confirms that inactivation of the VHL gene is not always required to develop CRCC, and that (an)other suppressor gene(s) on 3p could be involved. (3) We discard FHIT as an alternative pathway to VHL. We have not found new candidate regions along 3p by using a 1-Mb resolution array-based CGH. (4) The tumorigenesis mechanism of a second gastric tumor developed in the probandus is different from that of CRCC.
European Journal of Human Genetics advance online publication, 9 March 2005; doi:10.1038/sj.ejhg.5201371

Keywords: conventional renal cell carcinoma; three-step model; chromosome 3 translocations

Introduction

Hereditary conventional renal cell carcinoma (CRCC) is a rare entity mostly associated with the von Hippel-Lindau (VHL) syndrome caused by germline mutations in the VHL gene located in 3p25.[1–3] However, some hereditary CRCC cases associated with constitutional chromosome translocations involving chromosome 3 have also been reported.[4]

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Received 31 August 2004; revised 23 November 2004; accepted 1 December 2004

At the present time, seven families and two *de novo* cases carrying constitutional translocations involving chromosome 3 associated with high risk of developing CRCC have been reported.[5–14]

Although all CRCC-associated translocations involve chromosome 3, the partners are not recurrent. The positions of the breakpoints in chromosome 3 vary among all cases, but they all map in the proximal p- and q-arm regions. In some of them, different genes have been cloned at the breakpoints: FHIT and TRC8 on chromosomes 3 and 8, respectively, in t(3;8)(p14;q24);[15,16] DIRC3 and DIRC2 in t(2;3)(q35;q21) translocation;[17,18] DIRC1 on chromosome 2 in t(2;3)(q33;q21);[9] and finally, LSAMP and NORE1 in t(1;3)(q32;q13.3).[19] As regards t(3;8)(p14.1;p14.23), previously identified by our group,[14] no genes have been found in the breakpoints.[20]

A very common event identified by comparative genomic hybridization (CGH) or LOH in most of the tumor samples of these families is the loss of the derivative chromosome carrying the 3p segment. In addition to this, somatic VHL mutations have been identified in about 50% of tumor samples.[4,8,10,11,14,21–23]

According to these data and in contrast with the classical two-hit model of Knudson for tumor suppressor genes,[24] an alternative three-step tumorigenesis model for familial renal cell carcinoma with chromosome 3 translocation has been proposed.[22,25] The first step would consist of the inheritance of the constitutional chromosome 3 translocation that would be the primary oncogenic event. The second hit would be the loss in the tumor, of the derivative chromosome carrying the 3p segment der(3p), as a consequence of an erroneous chromatid separation, due to the presence of the translocation.[26] And finally, the third hit would occur in cells lacking the derivative chromosome and would consist of a somatic mutation in a tumor suppressor gene located on 3p, relevant to renal tumorigenesis, such as the VHL gene.

According to these points, hereditary CRCC associated with constitutional chromosome 3 translocations constitutes a paradigm from a genetic point of view with some unsolved questions: (1) Is the loss of der(3p) the first tumorigenic event? (2) Does the same tumorigenesis mechanism occur in other nonrenal tumors developed by translocation carriers? (3) Is a mutation in the VHL gene always necessary to develop the tumor?

In the present work, we have extended the analysis of a t(3;8)(p14.1;q24.23) family previously studied by us[14] to 36 members through four generations. We have performed an extensive genetic and cytogenetic analysis of the family: renal tumors and corresponding normal renal tissues, and a second gastric tumor developed by the probandus. Our results showed that: (1) previous to the loss of the derivative chromosome carrying 3p, another hit should occur to transform normal renal cells, (2) a gastric tumor developed by a translocation carrier does not follow the same multistep tumorigenesis mechanism as dorenal tumors, and (3) the VHL gene is not always necessary for the development of a tumor.

Materials and methods

Patients and samples

Previously, we described a family with three CRCC and t(3;8)(p14;q23).[14] Since then, the study has been extended to other familial branches. The number of members identified as affected with CRCC and/or translocation carriers is represented in Figure 1. At present, nine members of the family belonging to four generations have developed CRCC and eight of them developed bilateral forms. The ages of onset vary from 25 to 82 years. Other neoplasias have occurred in this family: two gastric tumors (II-2 and IV-3), a central nervous system tumor (II-3), a papillary renal cell carcinoma (III-2), two lymphomas (III-5 and III-22), and two breast tumors (III-3 and III-21).

Peripheral blood samples from 36 members of the family were available for cytogenetic analysis. In addition, 10 CRCC samples from four family members (IV-3, IV-22, IV-31, and V-5) (five paraffin embedded, four frozen, one fresh), and one fresh normal kidney tissue (IV-22 (N)), were studied. We also obtained frozen material from the gastric tumor of the proband (IV-3). Five sporadic CRCC samples were used as controls for immunohistochemistry studies.

Informed consent was obtained from all the individuals studied.

Cytogenetic analyses

Cytogenetic analyses were carried out on metaphase spreads derived from cultured peripheral blood lymphocytes using standard cytogenetic procedures. A normal fresh renal tissue sample from patient IV-22 of the t(3;8)(p14.1;q24.23) family was disaggregated with collagenase and subsequently cultured in RPMI 1640 medium supplemented with 10% fetal serum and antibiotics. Metaphase spreads were prepared according to standard procedures. Karyotypes were described according to ISCN.[27]

DNA isolation from blood cells and from paraffin-embedded and frozen tissues

High-molecular-weight DNAs were directly extracted from peripheral blood cells using standard procedures of proteinase K/SDS treatment followed by phenol/chloroform extraction and ethanol precipitation.

With regard to tumors, prior to DNA extraction, tumor and normal tissue areas were selected via microscopic inspection. DNA was extracted from paraffin and frozen tissues using a proteinase K, phenol–chloroform, Phase Lock Gel™ Light (Eppendorf AG, Germany) and EtOH protocol.

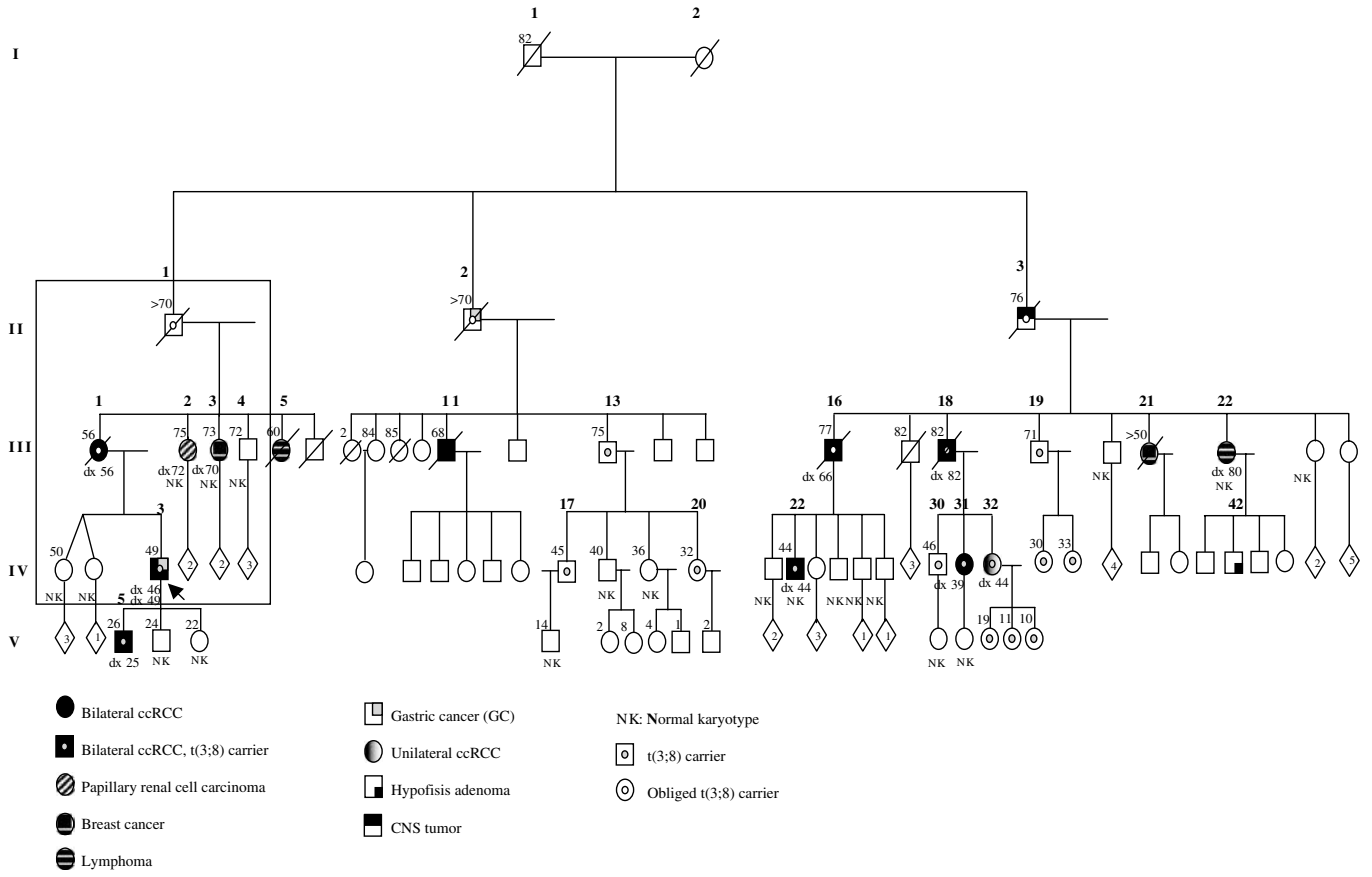

Figure 1 t(3;8)(p14.1;q24.23) family pedigree. The framed part of the pedigree was previously reported by Melendez *et al* (2003).

Comparative CGH and LOH analysis

For the analysis of the loss of der(3p), CGH was performed in paraffin-embedded and frozen samples, according to Kallioniemi *et al*[28] with slight modifications.

The 3p LOH analysis of the gastric tumor sample of patient IV-3 was performed with the microsatellites and conditions used by Melendez *et al*.[14]

Fluorescence *in situ* hybridization (FISH) on paraffin sections and on touch imprint cytologies

In order to detect the status of the derivative chromosome with the 3p segment in the tumor and normal renal tissue of the translocation carriers, FISH analyses were carried out on paraffin sections from IV-31 and on touch imprint cytology from IV-22. In each sample, an average of 110 (50–200) well-defined nuclei were analyzed. The tumor samples (both paraffin and touch cytology) contained more than 90% of tumor cells.

Two sets of probes were used to detect the loss of derivative chromosome 8 carrying the 3p segment: green commercial centromeric probe of chromosome 8 (CEP 8 (D8Z2) SpectrumGreen, Vysis) with red commercial probe of chromosome 17 as internal control (CEP 17 (D1721) SpectrumOrange, Vysis), and a green CEP 8 and a bacterial artificial chromosome that hybridized with 3p (RP11-129B22), labeled by nick translation with Texas Red (Vysis Inc., Downer's Grove, IL, USA).

Hybridization was carried out according to the manufacturer's instructions with slight modifications. The slides were deparaffinized, boiled in a pressure cooker with 1 mM EDTA (pH 8.0) for 10 min, and incubated with pepsin at 37°C for 30 min. The slides were then dehydrated. The probe was denatured at 75°C for 1 min before overnight hybridization at 37°C in a humid chamber. Slides were washed with 0.4 × SSC and 0.3% NP40.

Cell images were captured by using a cooled charge-coupled device (CCD) camera (Photometrics SenSys camera) connected to a computer running the Chromofluor image analysis system (Cytovision; Applied Imaging Ltd, New Castle, UK).

VHL gene analysis

Mutation analysis Direct sequencing of the three exons of the VHL gene was carried out.[29] Primers and conditions were as described elsewhere.[30]

Table 1 Multistep model in the origin and development of the tumors in a t(3;8) family

		Loss der(3p)		*VHL (tumor)*			*Immunohistochemistry*	
	Cases	*CGH*	*FISH*	*Mutation*	*Gross deletion*	*Methylation*	*VHL*	*FHIT*
Fam t(3;8)	IV-3 (L)	Yes	—	R161P[a]	—	—	−	−
	IV-3 (R)	Yes	—	L118P[a]	—	—	−	+
	IV-22	Yes	50%	No	No	No	+/−	+
	IV-22 (N)	No	0%	No	—	—	+	+
	IV-31	Yes	40–50%	No	No	No	−	−
	IV-31 (N)	No	0%	No	—	—	+	+
	V-5 (L)	Yes	—	CD123delG	—	—	−	NV
	V-5 (R)	Yes	—	No	No	No	+	NV
	IV-3 gastric	No	—	No	No	No	+	+

NV: not valuable (technical problems); —: not performed; No: no alterations; +: normal expression; −: no expression; +/−: reduced expression or in the limit of positivity/negativity; L: left; R: right; N: normal tissue.
[a]See Melendez *et al* (2003).

Methylation status of the VHL CpG islands, Southern blot, and multiplex ligation-dependent probe amplification (MLPA) The methylation status of the VHL CpG islands was analyzed by using the methylation-specific polymerase chain reaction (MSP).[31] The MSP technique applied for VHL gene has been previously described by us.[32]

Southern blot[33] and MLPA were used to detect great rearrangements in the VHL gene. In our work, these techniques were used for those tumor samples in which no VHL mutations were found. MLPA technique was performed by following the manufacturer's instructions using 200 ng of DNA (SALSA P016 VHL exon deletion test kit, MRC-Holland, Amsterdam).[34]

Immunohistochemistry

Sections were cut at a thickness of 3 μm, dried for 16 h at 56°C before being dewaxed in xylene, and rehydrated through a graded ethanol series to phosphate-buffered saline. Antigen retrieval was heat mediated in a pressure cooker treated for 2 min in a 10 mM citrate buffer (pH 6.5) with an additional proteinase K treatment (10 min at room temperature). Before staining the sections, endogenous peroxidase was blocked.

Immunohistochemical staining of FHIT (Zymed, San Francisco, USA) was performed at a dilution of 1:300 for 30 min at room temperature. After washing, the sections were incubated in a biotinylated secondary rabbit anti-rat (Dako, Glostrup, Denmark) for 30 min, followed by peroxidase-labeled streptavidin (Dako).

Immunohistochemical staining of VHL (NeoMarkers-Labvision, Fremont, CA, USA) was performed at a dilution of 1:10 for 40 min at room temperature. The Visualization System was EnVision (DakoCytomation, Glostrup, Denmark).

After incubation, sites of peroxidase activity were developed by using 3,3-diaminobenzidene chromogen as a substrate. Sections were counterstained with hematoxylin. A positive control was included with each batch of staining to ensure consistency between each consecutive run. Immunohistochemical staining was performed on an automated immunostainer (Techmate 500, Dako, Glostrup, Denmark).

A non-neoplastic kidney tissue adjacent to the tumor served as positive internal control.

Array-based CGH (aCGH)

A 1-Mb resolution aCGH[35] was used in order to explore possible microdeletions that could be taking place in the remaining 3p within those tumors without alterations in the VHL gene (IV-22, IV-35, and V-5 (R)).

Methods and conditions were as described previously.[35]

Results

A summary of the results is shown in Table 1.

Familial t(3;8)

At present, 36 living members have been studied to determine their translocation carrier status (Figure 1). Of these, 16 were demonstrated carriers and five deceased individuals were deduced to carry the familial translocation (Figure 2). Eight family members belonging to four generations (III-1, III-11, III-16, III-18, IV-3, IV-22, IV-31, and V-5) developed bilateral CRCC and only one, IV-32, developed unilateral CRCC, all of them being carriers of the constitutional translocation. Two carriers (II-2 and IV-3) presented gastric tumors. On the other hand, noncarrier family members also developed other types of tumors (papillary renal cell carcinoma, breast cancer, and lymphoma).

CGH and FISH analyses

We carried out a CGH analysis in renal tumor samples in order to detect copy number changes. The analysis of six tumoral DNA samples belonging to four individuals (two of IV-3, studied by Melendez *et al*,[14] IV-22, IV-31, and two of V-5) showed the loss of the derivative chromosome 8, which includes the 3p segment, der(3p), as the sole imbalance in all cases (Figure 2). No changes were observed

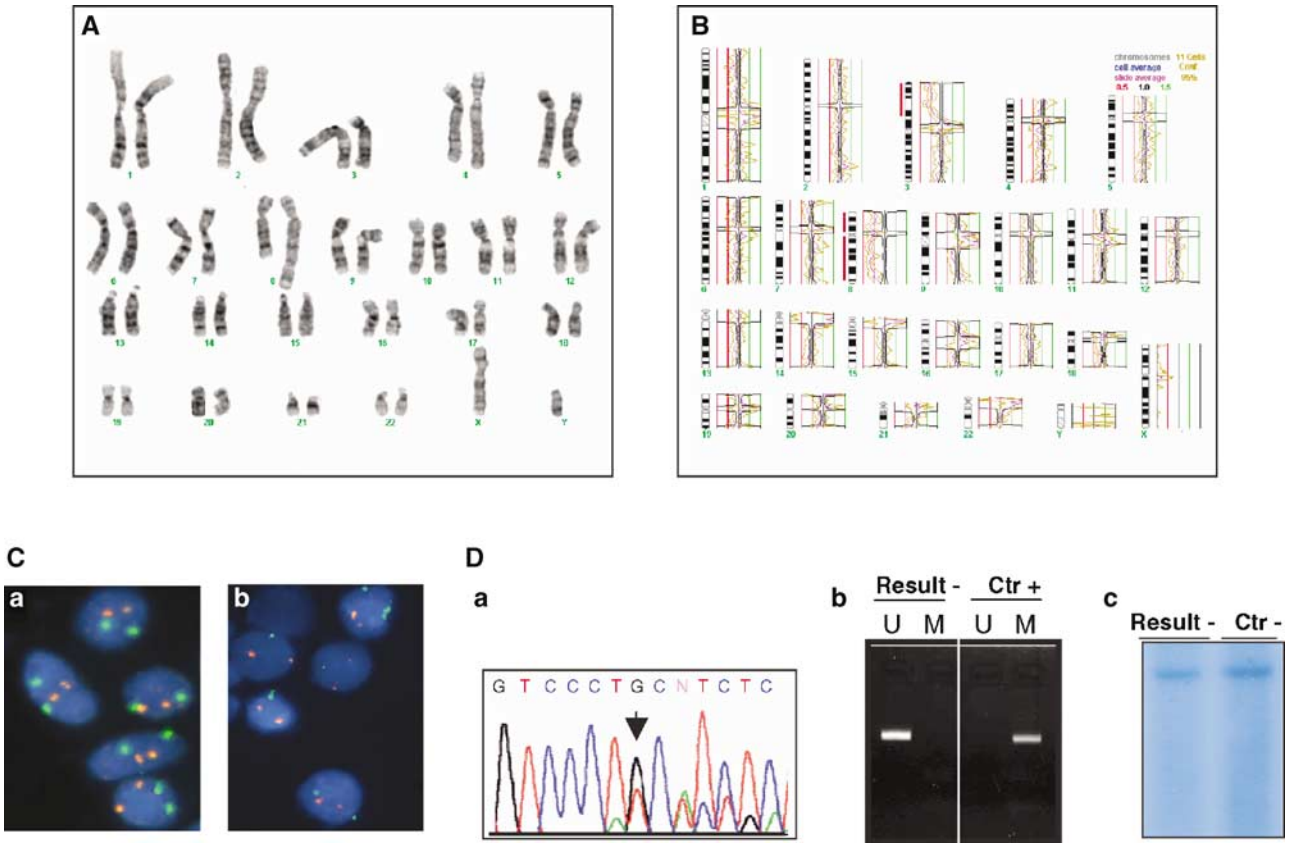

Figure 2 Multistep model in the t(3;8)(p14.1;q24.23) family. (**A**) Peripheral blood karyotype from a t(3;8) carrier. (**B**) Loss der(3p) in CRCC detected by CGH in all CRCC tumors. (**C**) FISH on touch imprint cytologies of normal and tumor samples (case IV-22). Green signal corresponds to CEP 8, and red signal to CEP 17. (**a**) No loss of der(3p), green signal, was observed in any of the normal renal cells analyzed. (**b**) Loss of one copy of CEP 8, loss of der(3p), was detected in approximately 50% of tumoral cells analyzed. (**D**)VHL gene studies in the tumors. (**a**) VHL mutation analysis: CD123delG detected in the tumor V-5(L). (**b**) Promoter hypermethylation analysis (IV-31); U = unmethylated, M = methylated. (**c**) Negative result of gross deletions studied by Southern blot (case IV-31).

in the normal kidney tissue from IV-22 and IV-31 members (Table 1). These results from the normal tissue were according to the normal karyotype obtained after culture from the fresh IV-22 (N) sample. The loss of der(3p) of tumor and normal samples was tested by means of FISH, and these showed that der(3p) loss was not present in all cells (around 50% of the nuclei), while this loss was not detected in the normal samples (Table 1 and Figure 2).

VHL

To verify the third hit of the proposed model for the development of CRCC in this family, a VHL gene mutational study on tumoral DNA was performed. We studied six tumor samples from four family members and we found three different VHL mutations (Table 1): D123delG, present in the tumor from patient V-5 (L), and R161P and L118P exhibited in two different samples from the same patient IV-3, as described by Meléndez *et al*.[14] The remaining three analyzed tumors from patients IV-22, IV-31, and V-5 (R) showed no VHL mutations, and neither did the normal tissues studied.

The next step in these three samples without VHL mutation was the detection of gross deletions (by Southern blot and/or MLPA), and the hypermethylation study of VHL by MSP technique. Neither gross deletion nor CpG island hypermethylation of the VHL gene were found (Table 1 and Figure 2).

In order to discard the fact that any other kind of mechanism of inactivation could be acting on the expression of VHL in these samples with no apparent genetic or epigenetic alterations, we performed an immunohistochemical analysis of the VHL protein. As expected, the three samples with VHL mutation showed lack of expression of the protein, and the three tumors with an apparently normal VHL gene showed a variable range of expression values: normal in the tumor of case V-5 (R), nonconclusive value in IV-22, and nondetectable in case IV-31 (Figure 3). Normal expression of the protein was observed in normal renal tissues (IV-22 (N)) and IV-31 (N)).

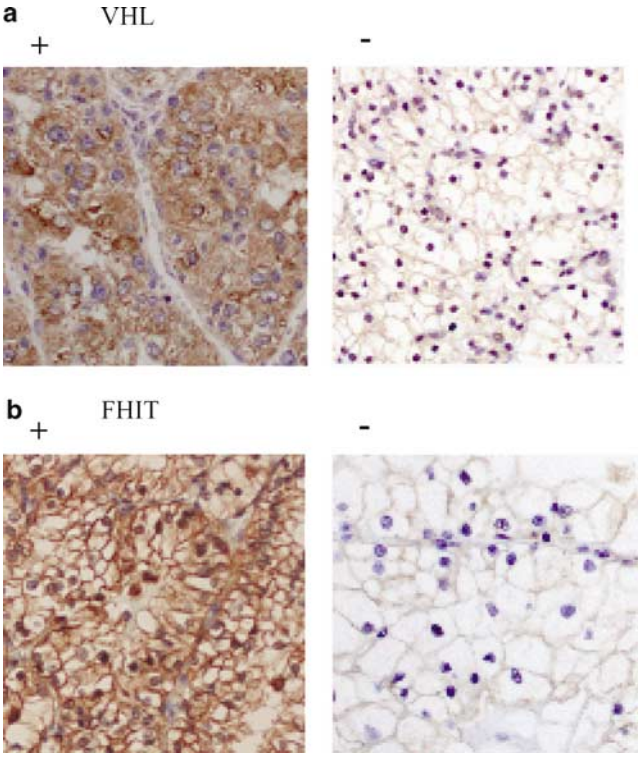

Figure 3 Immunohistochemistry of VHL and FHIT proteins. (**a**) VHL expression and lack of expression in cases V-5 (R) and IV-3 (L), respectively. (**b**) FHIT expression and lack of expression in cases IV-3 (R) and IV-31, respectively.

In order to test the accuracy of the immunohistochemistry in these kinds of tumors, we studied five sporadic CRCC: four out of five tumors carried mutation in the VHL gene and none of these expressed the protein, while the case without the VHL mutation did.

aCGH

To study if small microdeletions in the remaining 3p were taking place, we performed aCGH technique by using a 1-Mb resolution aCGH in three cases without VHL mutation (IV-22, IV-31, and V-5 (R)). Taking into account that not all tumoral cells lose der(3p), which includes part of 3p arm (p14.1→pter) and most of chromosome 8 (8pter→8q24.23), we considered the level of loss of chromosome 8 as the internal control of one loss.

Using this technique, we have not been able to distinguish homozygous microdeletions that could be affecting the nonlost 3p arm. BACs located in 3p were lost at the same level as those located in chromosome 8 (data not shown).

FHIT

We decided to study the expression of the FHIT protein in order to check its role as a tumor suppressor gene in families with chromosome 3 translocation and to determine whether FHIT could be an alternative pathway to that of VHL. Immunohistochemistry was performed on five samples: IV-3 (L), IV-3 (R), IV-22, IV-31, and V-5 (L). A wide variety of results were found as shown in Table 1: IV-3 (L) and V-5 (L) tumor samples with mutated VHL, showed the absence or reduction of FHIT expression, while IV-3 (R), also with VHL mutation, expressed the protein normally (Figure 3). On the other hand, the two samples studied without VHL alterations, IV-22 and IV-31, showed normal and no expression results, respectively. Expression of the protein was detected in normal renal tissues (IV-22 (N) and IV-31 (N)).

Other tumors

Patient IV-3 developed a gastric tumor at the age of 49, 3 years after having being diagnosed of bilateral CRCC. In addition to this, another member of the family (II-2), an obliged translocation carrier, died of gastric cancer. In order to prove or discard a possible relationship with the constitutional translocation, different studies were performed using DNA extracted from a frozen gastric tumor sample of IV-3 (Table 1). CGH analysis did not show loss of the chromosome derivative harboring the 3p segment and no alterations were detected in the VHL gene. LOH studies with molecular markers located on 3p and part of the q arm were also performed. The LOH results confirmed the results obtained by CGH in which no 3p loss was detected. We performed immunohistochemistry of VHL and FHIT proteins and both were expressed normally.

Discussion

Few families with hereditary CRCC and constitutional translocations involving chromosome 3p have been described. However, until now, no candidate genes have been cloned and an alternative model of three hits has been proposed in order to explain this atypical situation. This includes: (a) presence of the constitutional translocation, (b) loss of 3p in the tumor, and (c) mutation in a suppressor gene (VHL gene) in the remaining chromosome 3. In order to analyze this model in depth, and to give some answers to the questions above, we have extended our study in the family previously identified with three CRCC affected members and t(3;8)(14.1;q24.23).[14] Moreover, for this translocation, we cloned the breakpoints of the two involved chromosomes and we did not find genes in these regions.[20] In subsequent clinical controls and periodic advising, another seven cases of CRCC were diagnosed or reported. This allowed us to fully describe and analyze 36 members through four generations (Figure 1), and to study tumor and normal samples from the affected members.

In this family, we have observed high variability in the age of onset and an anticipation phenomenon through the generations: the three members of the II generation, obliged carriers of the t(3;8), died at over 70 years of age

and none of them had developed CRCC. In the following generation (III), the range of ages of onset is 56–82 years; in generation IV, 39–49; and in the last generation, one member has developed a bilateral form of the neoplasia at 25 years of age. The reason as to why this happens is not known, although some speculations have been proposed for other kinds of hereditary syndromes,[36] such as the role of genomic imprinting, or environmental interactions, as it has been observed for BRCA1 and two positive hereditary breast cancers.[37] In any case, the anticipation in the age of onset phenomenon is very important with regard to genetic counseling, in order to determine when these members should be studied to start the clinical follow-up.

In this large family, we have observed incomplete penetrance: two carriers in the III generation of 71 and 75 years of age have not yet developed CRCC and the three members of the II generation died over 70 without neoplasia. Although the risk of developing the bilateral form of CRCC is very high in these families, we cannot determine exact figures of risk on account of the few cases reported.

The three-step model in CRCC associated with t(3;8)(p14.1;q24.23): only three steps?

The three-step model previously proposed includes der(3p) loss in the tumoral samples as a second step. In our family, the six tumor samples analyzed by CGH presented der(3p) loss (Table 1), and the same phenomenon has been reported in most of the other chromosome 3 translocation families.[6,10–12,21,38] Including our data, more than 90% of the reported cases showed loss of the derivative chromosome carrying the 3p segment, confirming that 3p loss is a critical step in the genesis of CRCC. We investigated if the loss of der(3p) was also present in normal renal tissue by using FISH analysis in samples from IV-22 and IV-31 members. We demonstrated that normal cells did not show the der(3p) loss, by CGH and FISH, and that only a proportion of the tumor cells actually presented this aberration. The loss of der(3p) assessed by CGH in renal tumor samples was detected by FISH in around 50% of the nuclei (Table 1 and Figure 2). These results suggest that an additional genetic event should antecede the loss of 3p, playing a role in the normal to tumor transformation. This hypothesis was also suggested by Eleveld *et al* when they did not find the loss of der(3p) in two out of five CRCC samples belonging to a family with t(3;6)(q12;q15). So, tumorigenesis may be initiated in these families by other genetic and/or microenvironmental changes affecting chromosome stability.

The third step in this model includes the mutation of a suppressor gene located in the other normal chromosome 3. As VHL families develop CRCC in some cases, VHL gene was considered to be a good candidate and it has been found mutated in about 50% of the tumors of individuals with 3p translocations. In our tumor samples, VHL is only mutated in three of the six tumors (50%) (Table 1), a similar result to previous reports. In addition, we have not detected mutations in the normal renal tissues analyzed. To exclude other inactivation mechanisms affecting the VHL gene, we analyzed the presence of gross deletions and the methylation status in the three cases without mutation. For these three CRCCs, no genetic or epigenetic alterations in the VHL gene were identified, but immunohistochemistry revealed the lack of expression of the protein in at least one of the tumors (IV-31). In this case, another mechanism of inactivation, different from those we have studied, might be acting on the VHL gene. It is very significant that after different approaches, only 65% of tumors exhibited alterations involving VHL gene (4/6, including case IV-31). In fact, one of the tumors without apparent VHL alteration expressed normal level of the VHL protein. These results question the role of the VHL gene as a mandatory step in the development of CRCC, strongly supporting the existence of other notyet identified tumor suppressor genes located in 3p with much importance for the genesis of kidney tumors.

We studied the expression FHIT gene in order to check whether alterations in this gene could explain those cases without VHL alteration. The FHIT gene is located at chromosome 3p and was cloned in t(3;8)(p14;q24) of a family with CRCC.[39] Ohta *et al*[40] suggested that the FHIT gene would be a candidate for the initiation of familial and sporadic CRCC. However, the role of FHIT as a tumor suppressor gene is still controversial because (1) genomic deletions and point mutations are uncommon, (2) aberrant FHIT mRNA transcripts have also been identified in non-neoplastic tissues,[41–43] and (3) immunohistochemical studies have shown variable results among CRCC tumors.

Our results did not show any correlation between VHL and FHIT expression, indicating that FHIT does not seem to be an alternative pathway to VHL. We have also confirmed these results for sporadic cases (data not shown).

aCGH technique was performed in order to detect microdeletions in the remaining 3p in the cells that lose the der(3p) of the tumors without VHL alteration. In the three analyzed cases, we have not been able to detect homozygous microdeletions, but taking into account that the array used has a 1-Mb resolution, this could be making us miss the detection of deletions in the gaps. However, microdeletions as a second hit would be a very rare mechanism and it is difficult to think of it as a generalized event.

A gastric tumor developed by a translocation carrier does not follow the multistep tumorigenesis mechanism

Although the risk for developing bilateral CRCC is very high in this family (8/9 members), the risk of second tumors, different from CRCC, seems to be low, which is supported by similar observations in other chromosome 3

translocation families.[13] The majority of the members of the present family with other tumors were not carriers of translocation, except for the two members with gastric cancer. Member II-2, a compulsory translocation carrier, died of this tumor, and the probandus developed a gastric cancer at the age of 49 years, after bilateral CRCC. We studied this tumor but we found neither loss of the 3p by CGH nor VHL gene alteration, and FHIT expressed normally. These results suggest that, either this is a sporadic cancer that has coincided with a hereditary syndrome or that although related to the translocation, the tumorigenesis pathway is different from that of CRCC in the family. The last alternative could be supported by the early age of onset of the gastric cancer (49) and by the fact that another translocation carrier of this family died of this tumor. To our knowledge, in the other reported families with chromosome 3 translocations associated with high risk of CRCC, no other non-CRCC tumors developed by translocation carriers have been studied.[5,7,10,38] We consider that the study of other nonrenal tumors developed by translocation carriers would be useful to prove that the multistep tumorigenesis mechanism is specific of renal tumors.

In summary, in the large t(3;8) family that we have studied, we confirmed the multistep model previously proposed. However, based on our FISH data, we propose that a step previous to the loss of the 3p (genetic instability?) should happen in the origin of the tumor. A gastric tumor developed by a translocation carrier does not follow the same mechanism of tumorigenesis. Finally, our data question the role of VHL gene as a mandatory step in all CRCC cases, strongly supporting the existence of other not yet identified tumor suppressor genes located in 3p (we discard the FHIT gene as an alternative pathway to VHL, and microdeletions, studied by aCGH, as a possible mechanism of inactivation of a region in the remaining 3p). New studies analyzing other candidate genes on 3p will help to understand the genetic origin of these tumors.

Acknowledgements

This work was partially supported by the Consejería de Sanidad of the Autonomous Community of Madrid (CAM). Laura Valle holds a Fellowship from the CAM. We thank M[a] Carmen Carralero, M[a] Carmen Martín, and Rocío Letón for technical assistance, Marta Cuadros for her help in the LOH study, and Ellen Alacid for linguistic corrections.

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RESEARCH ARTICLE

A Haplotype Containing the *p53* Polymorphisms Ins16bp and Arg72Pro Modifies Cancer Risk in *BRCA2* Mutation Carriers

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Communicated by Dvorah Abeliovich

Germline mutations in the *BRCA1* and *BRCA2* genes confer a high lifetime risk of developing breast and other cancers; however, remarkable differences exist regarding disease manifestation in mutation carriers. It has been suggested that other genetic and/or environmental factors modify not only the appearance but also the age of onset and type of tumor in BRCA1/2-associated cases. The aim of the present study was to investigate the role of two *p53* polymorphisms (c.97-147ins16bp and c.215c>g, p.Arg72Pro) as potential modifiers. For this purpose we investigated the possible association between the two polymorphisms and disease status in 447 *BRCA1/2* mutation carriers belonging to 170 Spanish breast and/or ovarian cancer families. Genotype and haplotype analyses revealed that the presence of a specific haplotype carrying the allele without the 16-bp insertion and the variant allele for the Arg72Pro (No Ins-72Pro haplotype) was associated with an earlier age of onset in *BRCA2* mutation carriers. We found an increased risk of developing a first primary tumor (breast or ovarian) before 35 years of age for individuals who carried at least one No Ins-72Pro haplotype (OR: 2.69; 95% CI: 1.15–6.29; P = 0.022). We confirmed these data by a functional study in which we compared different *p53* genotypes in relation to their apoptotic response after cell treatment with a cytotoxic drug (AraC). Our results revealed a decrease in *p53* apoptotic rate associated with the No Ins-72Pro haplotype. Hum Mutat 0, 1–8, 2006.

KEY WORDS: modifier genes; p53; BRCA2

INTRODUCTION

Germline mutations in the *BRCA1* (MIM# 113705) and *BRCA2* (MIM# 600185) genes confer a high lifetime risk of developing breast and other cancers, and account for about 30% of hereditary breast and/or ovarian cancer families in most populations, including the Spanish population [Diez et al., 2003; Osorio et al., 2000]. Although these mutations are considered to be highly penetrant, estimates of the risk of developing breast cancer by age 70 vary from 36% to 70% depending on the family ascertainment and the population studied [Antoniou et al., 2003; Easton et al., 1995; Ford et al., 1998; Struewing et al., 1997; Thorlacius et al., 1998]. Other genetic and/or environmental factors, called "modifiers," are assumed to be the cause of these differences, in terms of both the risk of developing breast cancer and the appearance of other associated tumors. Although the identification of such factors is crucial to improve risk assessment in *BRCA1/2* mutation carriers, to date very few polymorphisms have been confirmed as genetic modifiers [Phelan, 1996] and [Rebbeck, 1999]. The last reported was the 5′UTR 135 g/c in the *RAD51* gene, which was confirmed by three independent studies to increase the risk for breast cancer specifically in *BRCA2* mutation carriers [Levy-Lahad, 2001; Wang, 2001; Kadouri, 2004].

Candidate modifiers include common polymorphisms in known cancer-related genes that by slightly altering the functionality of the encoded protein may increase or reduce the risk of developing cancer in individuals who already carry a highly deleterious mutation in *BRCA1* or *BRCA2*. In this sense, p53 is a good candidate because it has a critical role in cell cycle control, DNA repair, and apoptosis, and has been described as directly interacting with BRCA1 and BRCA2 in carrying out some of the functions

Received 30 June 2005; accepted revised manuscript 14 October 2005.

The Supplementary Material referred to in this article can be accessed at http://www.interscience.wiley.com/jpages/1059-7794/suppmat.

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Grant sponsor: Spanish Ministry of Science and Technology; Grant number: SAF03-02497; Grant sponsor: Spanish Ministry of Health; Grant number: FIS G03-179.

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DOI 10.1002/humu.20283

Published online in Wiley InterScience (www.interscience.wiley.com).

described above [Cheung et al., 2004; Jonkers et al., 2001; Ongusaha et al., 2003; Zhang et al., 1998]. *p53* (MIM♯ 191170) contains a variety of exonic and intronic polymorphisms, some of which, such as c.215c>g, p.Arg72Pro in exon 4 and a c.97-147ins16 bp in intron 3 (termed Ins16 bp), have been extensively tested as putative cancer susceptibility variants; however, not all studies have yielded consistent results [Helland et al., 1998; Kawajiri et al., 1993; Sjalander et al., 1996; Suspitsin et al., 2003; Wang-Gohrke et al., 1998; Weston et al., 1997]. In addition, some studies revealed evidence in favor of functional differences in apoptotic rates between the Arg and Pro variants [Biros et al., 2002; Dumont et al., 2003; Wu et al., 2002]. Association studies in the *p53* intron 3 16-bp insertion polymorphism also yielded controversial results. Some reported an elevated risk in patients with the intron 3 variant [Runnebaum et al., 1995; Wang-Gohrke et al., 2002], while other studies suggested that this polymorphism has a protective effect [Birgander et al., 1995; Sjalander et al., 1995]. Finally, the 72Pro allele was recently suggested as a possible *BRCA1/2* risk modifier, associated with an earlier breast cancer diagnosis in *BRCA1* mutation carrier women [Martin et al., 2003]. However, the number of cases in that study was too small for the authors to draw definite conclusions. All of these discrepancies may be related to the sample sizes, as well as to the necessity of analyzing the combined effects of the two polymorphisms in haplotypes rather than individually, as was pointed out in a recent publication [Gold et al., 2004].

The aim of the present study was to investigate the role of two *p53* polymorphisms (16-bp insertion and Arg72Pro) as *BRCA1* and/or *BRCA2* modifiers. For this purpose we investigated the possible association between the two polymorphisms and disease status in 447 Spanish *BRCA1/2* mutation carriers belonging to 170 breast/ovarian cancer families. To confirm our results we performed a functional assay to test whether functional differences existed in the apoptotic response of these p53 variants.

MATERIALS AND METHODS

Patients

A total of 447 *BRCA1* or *BRCA2* mutation carrier individuals, both affected and unaffected, were analyzed for the present study. The general phenotypic characteristics of the patients are described in Table 1. All of the patients were Spanish and therefore Caucasian. The carriers belonged to 170 breast and/or ovarian cancer families containing at least three women affected with breast and/or ovarian cancer (at least one of whom was diagnosed before 50 years of age), or at least one case of male breast cancer [Diez et al., 2003]. All individuals enrolled in the study carried a known deleterious mutation in *BRCA1* or *BRCA2*, detected by a combination of different techniques depending on the center of origin [Diez et al., 2003]. All carriers received genetic counseling and gave informed consent for genetic testing and use of their DNA samples for further research studies. None of the enrolled individuals reported having had prophylactic surgery as of the date they were enrolled in the study.

Determination of Genotypes and Haplotypes for p53 (GenBank:NM_000546.2) Polymorphisms

The genotypes for the exon 4 codon 72 Arg/Pro and intron 3 16-bp insertion polymorphisms (c.215c>g, p.Arg72Pro and c.97-147ins16bp, respectively) were determined for each sample. The genotypes were established with the use of PCR and restriction enzyme digestion. To analyze Arg72Pro, a fragment of 311 bp was amplified using primers 5′-TTTTCACCCATCTACAGTCCC-3′ forward and 5′-CTCCAGGGCAACTGACCGTG-3′ reverse. PCR was performed in a 25-µl final volume reaction containing 1 × PCR buffer, 0.2 mM of dNTPs, 10 pmoles of each primer, 50 ng of DNA, and 0.5 U of Taq polymerase under the following cycling conditions: 94°C for 5 min, followed by five cycles of 94°C for 30 sec, 68°C for 30 sec, and 72°C for 30 sec; five cycles at an annealing temperature of 65°C; and finally 25 cycles at an annealing temperature of 60°C with a final extension of 5 min at 72°C. After the PCR, 1 µl of the amplified product was digested with 10 U of BstUI overnight. The digestion products were separated by gel electrophoresis in a 3% agarose (Metaphor, MRC) gel and visualized by ethidium bromide staining. Homozygotes (Arg/Arg) produced a 175-bp and a 136-bp fragment, while mutant allele (Pro/Pro) homozygotes produced a single band of 311 bp, and heterozygotes (Arg/Pro) showed the three bands.

Ins16 bp was determined by PCR using primers 5′-CCATGGG ACTGACTTTCTGC-3′ forward and 5′-GGGGACTGTAGATG GGTGAA-3′ reverse, which amplify a 169-bp or a 153-bp fragment depending on the presence or absence of the 16-bp insertion, respectively. The PCR was carried out under similar conditions as for the codon 72 polymorphism, but with an annealing temperature of 60°C during the first five cycles, and 57°C for the remaining 30.

To establish haplotypes in cases that were heterozygous for both polymorphisms, an allele-specific PCR was designed. For this purpose the forward primer used to genotype the intron 3 variant was combined in different PCR reactions with two reverse primers 5′-GTGCAGGGGCCACGC-3′ and 5′-GTGCAGGGGCCACGG-3′ specific for the G (Arg) or C (Pro) alleles, respectively. Both PCRs were performed under the same cycling conditions, consisting of an initial denaturing of 5 min at 95°C followed by five cycles of 30 sec at 94°C, 30 sec at 65°C, and 30 sec at 72°C; five cycles with

TABLE 1. **Distribution of Carrier Individuals by Sex, Affection, and Altered Gene**

Disease status	*BRCA1* mutation carriers	*BRCA2* mutation carriers
Healthy	100 (32[a])	107(46)
Unilateral breast cancer	67	88 (6)
Bilateral breast cancer	20	10
Unilateral ovarian cancer	11	5
Bilateral ovarian cancer	5	–
Breast and ovarian cancer[b]	20	7
Other first primary tumour	1	6 (4)
Total	224 (32)	223 (56)

[a]Number of male carriers in parentheses.
[b]In this category are included all women affected first with breast cancer and then with ovarian cancer, without distinction between unilateral or bilateral for either tumour.

the annealing temperature changed to 60°C; 30 cycles with an annealing temperature of 57°C; and a final extension of 5 min at 72°C. Both the G- and C-specific PCRs generated a fragment of 281 bp when G or C was associated with the intron 3 without the 16-bp insertion, and a fragment of 297 bp when associated with the Ins16bp allele.

Apoptosis Assay

We performed a functional test to assess whether any difference existed among the different *p53* genotypes in response to apoptosis after treatment with the cytotoxic drug cytosine arabinoside (AraC) [Bonafe et al., 2002]. We selected 24 healthy donors from a transfusion center representing different genotypes, which included nine cases without any variant allele (No Ins-Arg72/No Ins-Arg72), six double heterozygotes (No Ins-Arg72/Ins16bp-72Pro), six cases carrying the 72Pro variant only (No Ins-72Pro/No Ins-72Pro), and three cases carrying both polymorphisms in homocigosis (Ins16bp-72Pro/Ins16bp-72Pro). Peripheral blood lymphocytes from these subjects were separated by Ficoll (Hystopaque) gradient centrifugation and then cultured in RPMI-1640 with 10% fetal bovine serum at a density of 10^6 cells/ml. Each sample was cultured in both the presence (5 μM) and absence of the cytotoxic drug AraC for 72 hr.

The level of apoptotic cells was measured using the Annexin-V-FLUOS staining kit (Roche) following the recommendations of the manufacturer, and then analyzed on a flow cytometer. Differences between apoptotic levels in the treated vs. untreated cultures from the same sample were used to compare among the different genotypes.

Statistical Analysis

The Hardy-Weinberg equilibrium for each polymorphism and haplotype distribution was tested using the likelihood ratio test. To analyze the association between any polymorphism/haplotype and the age at onset of the first primary tumor, we considered only female carriers, given that the number of male carriers was too low to reach statistically significant results. The outcome in affected women was defined as the age at diagnosis of the first malignancy (breast or ovarian), while in healthy carriers the outcome was the age at last follow-up or death. A first analysis was carried out using all female carrier family members using Huber and White robust estimators of variance to take into account any correlations between women from the same family [Hardin, 2001]. A second analysis was performed for 170 unrelated females, considering the youngest case (index case) in each family.

The possible association of polymorphisms and haplotypes with the age at onset of the first primary tumor was investigated in two ways. First, the "age at onset" was considered as a continuous variable, and differences were tested using linear regression. Second, the "age at onset" was considered as a dichotomous variable, as defined using the 25th percentile as the cutoff (tumors diagnosed in women younger than 35 were considered early-onset) and the analysis was performed using logistic regression. When the whole set of relatives was tested, unaffected women older than 34 were included as controls in the second analysis. For the analysis of haplotypes, the most frequently observed haplotype (No Ins-Arg72) was taken as a reference, and a dummy variable representing each other variant was included in the above-mentioned models.

Analyses were repeated separately for BRCA1- and BRCA2-positive individuals, and with breast cancer only considered as the tumor of interest. There were too few cases of other cancers (e.g., ovarian) to allow them to be considered separately. For the same reason, we could not perform a separate analysis among men.

To evaluate the functional differences between these polymorphisms, we compared different *p53* genotypes in relation to their apoptotic response after the cells were treated with AraC. We used a Kruskal-Wallis test to compare apoptotic rates among the genotypes.

RESULTS

We established genotypes and haplotypes for the two *p53* polymorphisms (Ins16 bp in intron 3, and Arg72Pro in exon 4) in 447 individuals who carried a deleterious mutation in either *BRCA1* or *BRCA2*. Table 1 shows the distribution by sex, affectation, and mutated gene of the individuals for whom we had complete clinical information. In Table 2 we show the genotype frequencies in the index case of the 170 families included in the analysis.

Risk Estimation for *p53* Genotypes and Haplotypes

The mean age at diagnosis of the probands was similar for *BRCA1* and *BRCA2* carriers (39 and 41 years, respectively). We estimated the risk of breast cancer for each *p53* polymorphism

TABLE 2. **Genotype Distributions of the Two *p53* Polymorphisms in the Index Cases of the 170 Families Analysed**

	c.215c>g, p.Arg72Pro[a]				c.97-147ins16bp[a]			
Group	**Arg72Arg**	**Arg72Pro**	**Pro72Pro**	**Total**	**No Ins/No Ins[a]**	**No Ins/Ins16bp**	**Ins16bp-Ins16bp**	**Total**
***BRCA1* mutation carriers**								
Observed	50 (60%)	29 (35%)	4 (5%)	83[b]	68 (80%)	15 (18%)	2 (2%)	85
Expected	49[c] (59%)	29.4 (35.4%)	4.6 (5.6%)		65.8 (77%)	18 (21%)	1.2 (2%)	
***BRCA2* mutation carriers**								
Observed	39 (55%)	24 (34%)	8 (11%)	71	54 (72%)	18 (24%)	3 (4%)	75
Expected	36.8[c] (52%)	28.6 (40.3%)	5.5 (7.7%)		53 (70%)	20 (27.4%)	2 (2.6%)	
Total[c]								
Observed	89 (58%)	53 (34%)	12 (7.8%)	154	122 (76%)	33 (21%)	5 (3%)	160
Expected	86.6 (56%)	57.7 (37.5%)	9.7 (6.5%)		118 (74%)	38.5 (24%)	3.5 (2%)	

[a]According to the cDNA sequence with accession number NM_000546.2. In the case of the Ins16-bp polymorphism, No Ins refers to the absence of 16-bp insertion.
[b]The total number of BRCA1 and BRCA2 index cases should be 90 and 80, respectively. The discordance observed with the total numbers in the table is because not all the individuals could be genotyped for the polymorphisms.
[c]The distribution of genotypes hardly deviated from those found for these polymorphisms in the Spanish general population. They were all in Hardy-Weinberg equilibrium. Only in the case of *BRCA2* mutation carriers we observed a nonsignificant slight deviation for the Arg72Pro, probably due to the further association found between the polymorphisms with and earlier onset of disease in this group.

by comparing the distribution of the different genotypes or haplotypes, with the absence of both variant alleles taken as the reference. We analyzed only female carriers and discarded those for whom complete clinical information was not available, and those in whom both haplotypes could not be determined unequivocally.

We performed a first analysis considering all female carriers, using Huber and White robust estimators of variance to correct for the relationship between members of the same family. No association was found between any genotype and appearance of the disease or a specific clinical presentation (Supplementary Table S1; available online at www.interscience.wiley.com/jpages/1059-7794/suppmat). However, while establishing the haplotypes, we found a higher frequency of the haplotype No Ins-72Pro, not carrying the 16-bp insertion in intron 3 and carrying the polymorphic allele in codon 72 (Pro), in subjects who developed their first primary tumor (breast or ovarian cancer) before 35 years of age (OR: 2.18; 95% CI: 1.16–4.09; P = 0.015; Table 3). The cutoff of 35 years was established using the 25th percentile, as described in Materials and Methods. When we analyzed *BRCA1* and *BRCA2* mutation carriers separately, we observed that the effect was a nonstatistically significant trend for *BRCA1* carriers (OR: 1.70; 95% CI: 0.62–4.66; P = 0.306), while for *BRCA2* carriers we found a more than 2.5-fold increased risk of developing the first tumor before 35 years of age for individuals carrying the No Ins-72Pro haplotype (OR: 2.69; 95% CI: 1.15–6.29; P = 0.022). The OR was obtained considering the presence of at least one No Ins-72Pro haplotype, because the number of subjects was too low to differentiate the effect of this haplotype in the heterozygous and homozygous states. We observed very similar results considering breast cancer cases only (OR: 2.18; 95% CI: 1.12–4.22; P = 0.021 for all carriers, and OR: 2.58, 95% CI: 1.04–6.41; P = 0.041 for *BRCA2* mutation carriers only; data not shown). We could not analyze the risk associated with ovarian cancer only, because the number of cases was too low.

We performed a second analysis considering the 170 index cases only. The results were consistent with the other analyses (OR: 2.36; 95% CI: 1.03–5.41; P = 0.043) considering *BRCA1* and *BRCA2* mutation carriers together.

Apoptotic Response of the p53 Variants

We tested whether the presence of the *p53* polymorphisms could have biological implications. For this purpose we compared the apoptotic response after treatment with a cytotoxic drug in a series of control individuals who carried the three most frequent *p53* haplotypes. The increase of apoptotic cells after treatment of peripheral blood lymphocytes compared with the apoptotic rate in untreated cells was measured for the different genotypes (Fig. 1). In general, we compared the behavior of the subjects without any variant in Arg72Pro or Ins16bp with those carrying at least one variant allele in either polymorphism. We found statistically significant variations in the apoptotic rates among the different genotypes, ranging from 5.72% to 54.19% (P = 0.023). The

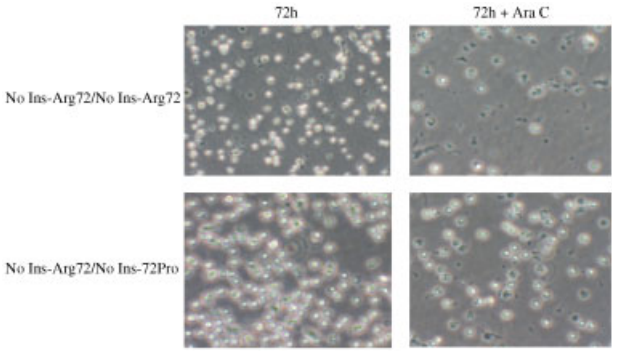

FIGURE 1. **Phase-contrast images of cells from individuals of two different genotypes after 72 hr of culture in the absence or presence of cytosine arabinoside. Differences in the amount of apoptotic cells detected in the presence or absence of the drug were considered as the apoptotic rate for comparison among genotypes.**

TABLE 3. **Relative Risks to Develop the First Primary Tumor Before 35 Years of Age, Associated with the Presence of the Different c.97-147Ins16bp and c.215c>g, p.Arg72Pro Haplotypes, and Considering All Carriers**

Haplotype	Healthy relatives ≥ = 35 years[c] (n = 60)	Cases with age of onset ≥ = 35 years (n = 157)	Cases with age of onset ≤35 years (n = 48)	OR[d]	95% CI	*P*-value
All carrier women[a]						
No Ins-Arg72/No Ins-arg72[b]	41	92	27	1.00		
No Ins-72Pro[e]	**8**	**32**	**16**	**2.18**	**1.16–4.09**	**0.015**
Ins16bp-Arg72	1	7	2	1.01	0.28–3.62	0.988
Ins16bp-72Pro	13	32	7	0.68	0.27–1.69	0.402
***BRCA1* mutation carriers**	(n = 36)	(n = 84)	(n = 26)			
No Ins-Arg72/No Ins-Arg72	24	51	17	1.00		
No Ins-72Pro	3	17	7	1.70	0.62–4.66	0.306
Ins16bp-Arg72	0	2	1	2.40	0.28–20.5	0.424
Ins16bp-72Pro	10	17	3	0.49	0.13–1.85	0.292
***BRCA2* mutation carriers**	(n = 24)	(n = 73)	(n = 22)			
No Ins-Arg72/No Ins-arg72	17	41	10	1.00		
No Ins-72Pro	**5**	**15**	**9**	**2.69**	**1.15–6.29**	**0.022**
Ins16bp-Arg72	1	5	1	0.66	0.16–3.16	0.602
Ins16bp-72Pro	3	15	4	0.86	0.22–3.34	0.831

[a]We only considered carrier women for the analysis; the analysis should be made separately for each sex and the number of affected male carriers was too low.
[b]We considered this as the reference group containing all the women homozygous for the haplotype with the two normal alleles for each of the polymorphisms.
[c]Healthy carriers younger than 35 years of age were not included in the analysis.
[d]ORs were calculated by comparing the haplotype frequencies between the group of healthy carriers ≥35 years plus cases with age of onset ≥35 years, and the group of cases with age of onset <35 years.
[e]In this group we included all the women carrying at least one haplotype containing the variant allele for the Arg72Pro polymorphisms and not the 16-bp insertion. The low number of cases did not allow us analyze homozygotes and heterozygotes separately for this haplotype.

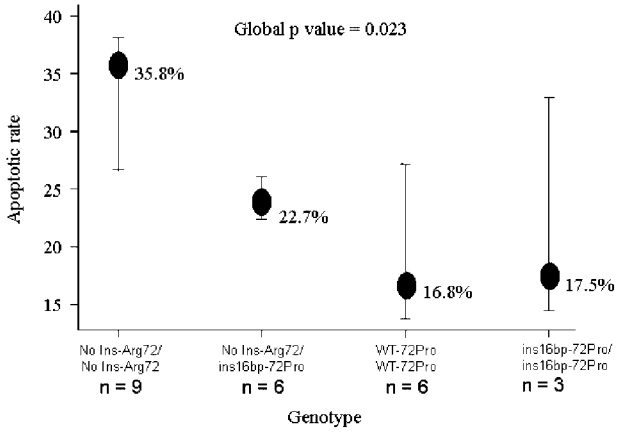

FIGURE 2. **Comparison of the apoptotic rates among different *p53* combined genotypes. The median apoptotic rates and 25 and 75 percentiles are represented for each genotype. Genotypes harboring the codon 72 Pro (C) allele showed lower apoptotic rates than those with codon 72 Arg (G) alleles.**

highest level of apoptosis was found in the No Ins-Arg72/No Ins-Arg72 subjects, with a median rate of apoptosis of 35.8% (Fig. 2). In general, individuals who carried at least one mutant 72Pro allele showed lower apoptotic indexes: 17.5% for No Ins-72Pro/No Ins-72-Pro, 16.8% for No Ins-Arg72/No Ins-72Pro, and 22.7% for the double heterozygotes No Ins-Arg72/Ins16bp-72Pro (Fig. 2).

DISCUSSION

Modifier genes are assumed to contribute to the interindividual phenotypic differences observed among *BRCA1/BRCA2* mutation carriers. These differences affect not only the appearance of the disease, but also the type of tumor and the age at onset. Although the identification of such factors is crucial for improving risk assessment, to date only one polymorphism in the *RAD51* gene has been confirmed by independent studies to modify cancer risk in *BRCA2* mutation carriers [Kadouri et al., 2004; Levy-Lahad et al., 2001; Wang et al., 2001]. In the present study we evaluated the candidate polymorphisms Arg72Pro and Ins16bp in the *p53* gene as genetic modifiers of *BRCA1* and *BRCA2* penetrance. For this purpose we analyzed 447 *BRCA1* or *BRCA2* mutation carriers belonging to 170 Spanish breast and/or ovarian cancer families. The analysis of genotype and haplotype frequency distributions in all available female carriers showed that a specific haplotype containing the consensus sequence and variant alleles for Ins16bp and Arg72Pro, respectively, termed No Ins-72Pro, was more frequent in subjects who developed their first primary tumor (breast or ovarian) before 35 years of age (OR: 2.18; 95% CI: 1.16–4.09; P = 0.015; Table 3). When we analyzed *BRCA1* and *BRCA2* mutation carriers separately, we found that the increased risk was most apparent in BRCA2 cases (OR: 2.69; 95% CI: 1.15–6.29; P = 0.022), while in BRCA1 carriers it was only a trend. When we performed the same analysis but considered breast cancer cases only, we still obtained statistically significant results (OR: 2.18; 95% CI: 1.12–4.22; P = 0.021 for all carriers, and OR: 2.58; 95% CI: 1.04–6.41; P = 0.041 for *BRCA2* mutation carriers), suggesting that most of the observed effect came from the increased risk for breast cancer.

A second analysis considering only 170 index cases (unrelated), achieved similar results. The reduction in statistical significance was expected, given the decreased number of subjects analyzed.

Several epidemiological studies have suggested an association of the codon 72 Pro allele with an increased risk of breast cancer and other major cancer types; however, in other studies statistical significance was not reached [Birgander et al., 1995; Sjalander et al., 1995, 1996; Weston et al., 1997; Wu et al., 2002]. In the case of Ins16bp, the results are even more contradictory, as there are reports of both deleterious and protective effects of the polymorphism [Birgander et al., 1995; Runnebaum et al., 1995; Sjalander et al., 1995; Wang-Gohrke et al., 2002]. The discrepancies observed regarding the risk associated with these two polymorphisms could be due to, among other factors, the necessity of studying haplotypes rather than individual variants. In the present study we only detected an effect when we considered both polymorphisms combined in haplotypes. The No Ins-72Pro haplotype was associated with an earlier age at onset in *BRCA2* mutation carriers, which would confirm the suggested risk effect for this polymorphism, but only when the Ins16bp is not present on the same chromosome, which suggests a "protective" effect for the latter polymorphism.

In addition to our results from the risk estimate analysis, our experimental data regarding the apoptotic response show that the No Ins-72Pro haplotype may exert a functional effect. Previous studies reported functional differences between the Arg and Pro alleles in codon 72. It seems that the Pro allele is less efficient in suppressing cellular transformation [Thomas et al., 1999] and inducing apoptosis in response to several stimuli, such as γ-rays or cytotoxic drugs [Biros et al., 2002; Bonafe et al., 2002; Dumont et al., 2003; Wu et al., 2002]. However, other reports have suggested that 72Pro is a stronger activator of transcription than Arg72 [Thomas et al., 1999]. Most of the functional assays have searched for differences in the codon 72 variants performed under different in vitro conditions, but again it is possible that an interaction between the different *p53* polymorphisms could modify the effect of a specific variant. We observed that the response to apoptosis of different *p53* genotypes involving the codon 72 and the intron 3 polymorphisms that varied, depending on the haplotype (Fig. 1). In general, significant differences in the response to apoptosis were found among the different *p53* diplotypes (P = 0.023; Fig. 2). Homozygous carriers for the No Ins-Arg72 haplotype appeared to be more efficient in inducing apoptosis than those with genotypes including at least one mutant 72Pro-allele. Within this second group, it is also noteworthy that the most reduced apoptotic indexes were found to be associated with individuals who did not harbor intron 3 16-bp insertion alleles (No Ins-72Pro/No Ins-72-Pro and No Ins-Arg72/No Ins-72Pro), while those subjects in whom the 72Pro allele appeared in conjunction with the Ins16bp allele (No Ins-Arg72/Ins16bp-72Pro) showed an intermediate apoptotic rate (Fig. 2). These functional results are in complete agreement with our risk estimation study.

A modifier effect of *p53* in *BRCA2* penetrance is not difficult to justify, given the functional interaction of p53 with both BRCA1 and BRCA2 [Jonkers et al., 2001; Ongusaha et al., 2003; Zhang et al., 1998]. The relationship among these genes is confirmed by the fact that *p53* is mutated at a significantly higher frequency in breast carcinomas arising in carriers of germline *BRCA1* and *BRCA2* mutations than in sporadic carcinomas [Gasco et al., 2003]. With regard to *BRCA2*, it has been suggested that Brca2 deficiency and downregulation of p53 can jointly promote mammary tumorigenesis [Cheung et al., 2004]. Thus, it seems that the lack of function of BRCA2, combined with reduced effectiveness of p53 (caused by a germline mutation and a specific haplotype, respectively) would promote an earlier appearance of the tumor.

Why might p53 modify BRCA2 and not BRCA1? The reason may be related simply to the specific functional relationships between these proteins, but it may also have to do with the lower penetrance of *BRCA2* mutations [Ford et al., 1998]. In this case, slight genetic or environmental changes could result in altered disease manifestations (i.e., an earlier age at diagnosis) more easily in *BRCA2* mutation carriers than in *BRCA1* mutation carriers. It is noteworthy that the same differential effect among *BRCA2* mutation carriers has been observed for the other reported genetic modifier, 135 G/C in the *RAD51* gene [Kadouri et al., 2004; Levy-Lahad et al., 2001; Wang et al., 2001]. With regard to *BRCA1*, it is possible that an effect also exists, as previously suggested [Martin et al., 2003]; however, in the present study we could not achieve statistically significant results.

In conclusion, we have found that the specific haplotype No Ins-72Pro in *p53* modifies cancer risk in *BRCA2* mutation carriers, and confers an increased risk of developing a first primary tumor before 35 years of age. These results are supported by the confirmation that the No Ins-72Pro haplotype has a functional effect: homozygous carriers for the No Ins-Arg72 haplotype are more efficient at inducing apoptosis than those with genotypes that include at least one mutant 72Pro-allele. Our findings constitute the second report of a BRCA2 genetic modifier, and may be very important for improving genetic assessments. If our results are confirmed, the knowledge of an increased risk of developing the disease before 35 years of age in healthy *BRCA2* mutation carriers could lead to the modification of prevention and follow-up strategies for these specific women.

ACKNOWLEDGMENTS

We thank Rocío Letón and Alicia Barroso for excellent technical assistance, and Roger Milne for reviewing the manuscript.

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Immunohistochemical classification of non-*BRCA1/2* tumors identifies different groups that demonstrate the heterogeneity of *BRCAX* families

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Around 25% of hereditary breast and ovarian cancer families have mutations in the *BRCA1* and *BRCA2* genes. The search for other genes has until now failed, probably because there is not one single *BRCAX* gene, but rather various genes that may each be responsible for a small number of breast cancer families and/or may interact according to a polygenic model. We have studied 50 tumors from probands belonging to non-*BRCA1/2* breast cancer families (*BRCAX*), using 25 immunohistochemical markers. The objective was to classify these tumors and confirm that they are heterogeneous. Unsupervised cluster analysis showed the existence of the following two main groups of tumors: high-grade and estrogen receptor (ER)-negative tumors (50%), and low-grade and ER-positive tumors (50%). In addition we identified five subgroups, three among the high-grade and two among the low-grade groups; one overexpressing HER-2 (18%); one with a basal-like phenotype (14%); one with a normal breast-like phenotype (18%); a luminal A subgroup (36%), and a luminal B subgroup (14%). Hypermethylation of the *BRCA1* gene was observed in 42% of the cases, spread across all five subgroups, but only 37% of those had loss of heterozygosity as well. These latter cases were all clustered in the high-grade group and the majority of them in the basal-like subgroup. Our results show that familial non-*BRCA1/2* tumors are heterogeneous and suggest a polygenic model for explaining the majority of *BRCAX* families. In addition we have defined a subset of them that have somatic inactivation of the *BRCA1* gene.
Modern Pathology advance online publication, 21 September 2007; doi:10.1038/modpathol.3800969

Keywords: BRCAX; breast cancer; classification; hereditary; hypermethylation immunohistochemistry; LOH

More than a decade has passed since *BRCA1* and *BRCA2* were cloned[1,2] and their association with familial breast and ovarian cancer (FBOC) was established.[3] However, recent data indicate that these two genes explain only 25% of these families.[4–6]

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Received 13 May 2007; revised 20 August 2007; accepted 5 September 2007; published online 21 September 2007

The large number of families without an identified causative gene mutation has led many a groups to pursue putative *BRCAX* gene(s) through different approaches, but without success. Several reports have been published since 1995 suggesting linkage of the *BRCAX* gene to specific chromosomal regions,[7,8] but these data have not been reproduced in larger series.[9–11] Hedenfalk *et al*[12] carried out a study using expression arrays in a small group of non-*BRCA1/2* tumors, and concluded that they were heterogeneous and could be split into two main groups; but again, these results have not been reproduced. It has also been suggested that *BRCAX* families could be explained by a polygenic model,

or that they might carry mutations in another gene or genes conferring a moderately increased risk of breast cancer.[13–15]

Using paraffin-embedded tissue, we and others have previously demonstrated that *BRCA1* and *BRCA2* tumors can be differentiated because they have a specific immunohistochemical profile.[16–20] Based on these results, we hypothesized that the use of different immunohistochemical markers might help to group the non-*BRCA1/2* tumors, and to confirm that they are heterogeneous. The confirmation of this heterogeneity and their classification would be very important for further studies searching for candidate genes.

Materials and methods

Patients

We collected paraffin-embedded tumor tissues from 50 individuals (mean age 47 years) from 50 different high-risk families, who were studied for mutations in the *BRCA1* and *BRCA2* genes. These individuals were from families with at least three members affected with breast and/or ovarian cancer, at least one of whom was younger than 50 when diagnosed.[4] All 50 individuals were screened for mutations, including large deletions, in the *BRCA1* and *BRCA2* genes, and no mutations were detected.[4,21] The complete coding sequence and exon–intron boundaries of the BRCA1 and BRCA2 genes were analyzed by a combination of the following different techniques, depending on the center of origin: SSCP, PTT, CSGE, DGGE, and direct sequencing.[21]

We compared the profiles of non-*BRCA1/2* tumors *vs* a group of 33 tumors from patients carrying a mutation in the *BRCA1* gene, selected using the same criteria and studied with the same methodology as described above. Finally, we included a control group of 50 sporadic tumors that were selected because they were diagnosed at similar ages to the non-*BRCA1/2* tumors (mean 49 years). In order to confirm that the non-*BRCA1/2* and sporadic cases were genetically different, we estimated *BRCA1* and *BRCA2* carrier probabilities for both the groups using BRCAPRO.[22] Data on the majority of the markers assessed in the present study have been previously reported for all three groups of tumors.[18]

Tissue Microarray Construction

The morphological subtype and grade was assessed in complete sections of each tumor stained with hematoxylin–eosin (H–E). The non-*BRCA1/2* tumors consisted of 44 invasive ductal carcinomas, five *in situ* ductal carcinomas and one invasive lobular carcinoma.

Representative areas of the different lesions were carefully selected on H–E sections and marked on individual paraffin blocks. Two tissue cores (1 mm in diameter) were obtained from each specimen. In addition, four cores of normal breast tissue and two cores of tonsil were included as controls. The tissue cores were arrayed onto one independent new paraffin block using a tissue microarray (TMA) workstation (Beecher Instruments, Silver Spring, MD, USA). The final TMA consisted of 106 cores, each 1 mm in diameter, spaced 0.8 mm from each other. A section stained with H–E was studied to confirm the presence of morphologically representative areas of the original lesions. The *BRCA1* and sporadic tumors were included in two separate TMAs using the same technique.

Immunohistochemical Studies

Immunohistochemical staining was performed by the Envision method (Dako, Glostrup, Denmark), with a heat-induced antigen retrieval step. Sections from the tissue array were immersed in 10 mM boiling sodium citrate at pH 6.5 for 2 min in a pressure cooker. Antibodies, dilutions and suppliers are listed in Table 1.

Between 150 and 200 cells per core were scored for the percentage of positive nuclei or cytoplasms, depending on the marker. We evaluated nuclear staining for estrogen receptor (ER); progesterone receptor (PR); p53; Ki-67; cyclins D1, D3, E, and A; p16; p27; p21; Skp2; retinoblastoma protein (Rb); E2F6; MDM2; topoisomerase IIα; survivin; and CHEK2, and evaluated cytoplasmic staining for BCL2, vimentin, cytokeratin 5/6 (CK5/6), cytokeratin 8 (CK8), and cyclin B1, as previously described.[16–18] Only the percentage of stained cells was considered (independent of the intensity), and the positivity threshold for each marker is listed in Table 1. We and others have previously used the same threshold in the analysis of these markers.[16,18,20,23–27] HER-2 expression was evaluated according to the four-category (0–3+) DAKO system proposed for the evaluation of the HercepTest, and HER-2 expression of 3+ was the only value considered positive, as previously described.[18,19]

Statistical Analysis

Hierarchical unsupervised cluster analysis was performed by means of the UPGMA (unweighted pair-group method using arithmetic averages) method using correlation distance and Euclidean distance between markers. The cluster was displayed using SOTAARRAY[28] (software available at http://gepas.bioinfo.cipf.es/). The method was implemented in the GEPAS package.[29] Immunohistochemical results were represented by a range of color from green to red, the most green representing the lowest, and the most red the highest percentage of positive cells for each marker. Exceptions were grades which were scaled as 33% 'expressed' for grade 1, 66% for grade 2, and 100% for grade 3 and HER-2, which

Table 1 Antibodies used in the present immunohistochemical study and threshold to consider a tumor as positive, used in χ^2 analysis

Antibody	*Clone*	*Dilution*	*Supplier*	*Threshold (%)*
ER	1D5	1:30	Novocastra	10
PR	1A6	1:30	Novocastra	10
BCL2	124	1:80	DAKO	70
Ki-67	MIB1	1:30	DAKO	0–5/6–25/>25
p53	DO-7	1:50	Novocastra	25
HER-2	Herceptest	Prediluted	DAKO	3+
Cyclin D1	DCS-6	1:100	DAKO	30
Cyclin D3	DCS-22	1:10	Novocastra	[a]
Cyclin E	13A3	1:10	Novocastra	[a]
Cyclin A	6E6	1:100	Novocastra	[a]
Cyclin B1	7A9	1:25	Novocastra	[a]
p21	EA10	1:50	Oncogene	[a]
p16	Poly mouse	1:50	Santa Cruz	50
p27	57	1:1000	Transduction Lab	50
Skp2	1G12E9	1:10	ZYMED	[a]
Rb	G3–245	1:250	BD PharMingen	[a]
E2F6	Poly goat	1:50	Santa Cruz	[a]
CHEK2	DCS-270	1:25	Novocastra	60
Topoisomerase IIα	Ki-S1	1:400	DAKO	[a]
MDM2	IF2	1:10	Oncogene	[a]
CK 5/6	D5/16 B4	1:25	DAKO	[a]
CK 8	35BH11	1:10	DAKO	80
Vimentin	V9D	1:500	DAKO	[a]
Survivin	Poly rabbit	1:1000	RD Systems	[a]
EGFR	EGFR.113	1:10	Novocastra	[a]

ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; EGFR, epidermal growth factor receptor.
[a]Any positive cell.

was scaled as for 100% for positive (3+), and 0% for negative (Figure 1). We used the CAAT software based on Silhouette Width for clustering validation (software available at http://gepas.bioinfo.cipf.es/).

The χ^2-test was performed to determine the differences in the distributions of the expression of each antibody and grade between the groups (Tables 2 and 3). The statistical program SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for this analysis. Correction for multiple testing was made by a permutation method in which group membership was randomly assigned, conserving observed proportions, and the distribution of minimum *P*-values determined over 10 000 permutations. Differences in median BRCAPRO probabilities were tested using the Mann–Whitney rank-sum test.

BRCA1 Promoter Hypermethylation

DNA methylation patterns in the CpG islands of the promoter of the *BRCA1* gene were determined by methylation-specific PCR in primary tumors after bisulfite treatment of DNA.[30] Placental DNA treated *in vitro* with *Sss*I bacterial methylase was used as a positive control, and DNA from normal lymphocytes was used as a negative control for methylated alleles of *BRCA1*.

BRCA1 Loss of Heterozygosity

Loss of Heterozygosity (LOH) analysis of the *BRCA1* gene was performed using the intronic microsatellite markers D17S1322 and D17S855 that localize to introns 19 and 20, respectively, and D17S1327 that localizes in 17q21.31 outside the *BRCA1* gene.[31] The forward primer for each set was labeled using the fluorescent dye FAM (Applied Biosystems/PE Biosystems, Foster City, CA, USA). Reactions were cycled as follows: 95°C for 5 min, then 35 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 90 s, followed by final elongation at 72°C for 5 min.

Allele sizes were determined using an automated capillary sequencer (ABI Prism™ 310; Applied Biosystems, Perkin Elmer, Warrington, UK) and were analyzed using GeneScan 3.1 software (Applied Biosystems, Warrington, UK). LOH was determined when the difference between peaks representing alleles in the tumor and the corresponding normal DNA reactions exceeded 25%.

Results

The morphological and immunohistochemical profiles of non-*BRCA1/2* tumors were established by analyzing grade and 25 immunohistochemical markers in 50 such tumors, and comparing them with 50 sporadic tumors. Non-*BRCA1/2* tumors were of lower grade (adjusted $P=0.04$); 54% were grade 1 *vs* 20% of sporadic tumors (Table 2). Although there was marginal evidence that p53 and p21 expression differed between the two groups, these associations disappeared after correction for multiple testing. Overall, the expression of markers related to proliferation, cell cycle, apoptosis, hormone recep-

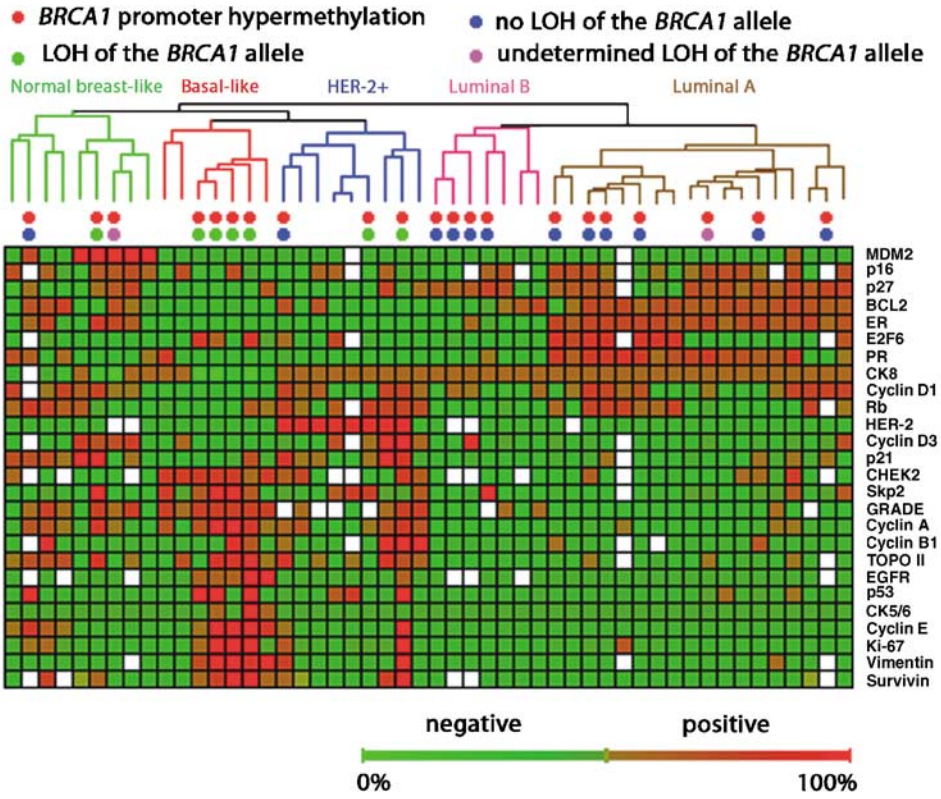

Figure 1 Hierarchical clustering of 50 non-BRCA1/2 tumors. White squares correspond to data not available. The percentage of positive cells for each immunohistochemical marker is represented as a range of color between the most green (lowest percentage) and the most red (highest percentage). Intermediate colors represent percentages between the lowest and the highest.

tors, and epithelial proteins showed similar patterns after adjustment for multiple testing (Table 2).

Although the phenotype of both groups was similar, they were genetically very different. Using BRCAPRO we found that the median probability of not being carrier of *BRCA1* or *BRCA2* mutations was 0.994 (range 0.964–0.997) for the sporadic tumors and 0.519 (range 0.062–0.987) for the non-*BRCA1/2* tumors ($P<0.0001$).

Hierarchical Unsupervised Cluster Analysis

We performed a hierarchical unsupervised cluster analysis of the non-*BRCA1/2* tumors based on the 25 immunohistochemical markers and grade, and found that the 50 tumor samples were separated into two main groups with 25 tumors each, differentiated primarily by grade and ER status. The high-grade branch (Figure 1, left) included tumors of grade 2 or 3 (brown and red squares, respectively) that were ER negative and had overexpression of proteins that promote cell cycle progression and proliferation. The low-grade branch (Figure 1, right) included grade 1 tumors (green squares) that were ER positive and showed overexpression of proteins related to the inhibition of the cyclin–CDK complexes, or the overexpression of luminal epithelial proteins such as CK8.

Within the high-grade branch we distinguished the following three different subgroups: one characterized by HER-2 overexpression (Figure 1, blue branch) that corresponded to 18% of all cases; a second basal-like subgroup containing 14% of the cases; and a third group that represents 18% of all cases, defined by low expression of the luminal epithelial marker CK8 and overexpression of other proteins associated with cell cycle progression and proliferation. Furthermore, this latter subgroup contained most of the ER-positive tumors in this predominantly ER-negative branch (Figure 1, green branch), and we have named it the 'normal breast-like' group because of its similarity to the group described by Sorlie *et al*[32] with this name.

The low-grade branch could be divided into two further subgroups (Figure 1, pink and brown branches). The brown branch in Figure 1 was the largest (18 tumors) and demonstrated higher expression of ER, BCL2, CK8, and proteins that inhibit cell cycle progression. The second subgroup contained the remaining seven (14%) cases (Figure 1, pink branch) and showed low, or loss of, expression of ER, PR, and BCL2, but conserved the expression of CK8. We have named them the luminal A and B groups, respectively, according to Sorlie's classification.[32]

We performed an unsupervised cluster analysis with the 50 sporadic breast tumors, using the same

Table 2 Comparison between non-*BRCA1/2* tumors and a group of sporadic breast carcinomas of the number (and percentage in parenthesis) of positive cases for each immunohistochemical marker, unless otherwise indicated

	Non-BRCA1/2 (n = *50*) *(%)*	P	*Sporadic* (n = *50*) *(%)*
Grade			
1	24 (54.5)		10 (20.0)
2	9 (20.5)		17 (34.0)
3	11 (25.0)	**0.002**[a]	23 (46.0)
Ki-67			
0–5%	35 (70.0)		26 (53.1)
6–25%	9 (18.0)		18 (36.7)
>25%	6 (12.0)	0.1	5 (10.2)
ER	30 (60.0)	0.3	33 (68.8)
PR	28 (56.0)	0.8	27 (55.1)
BCL2	23 (46.0)	0.8	22 (45.8)
p53	7 (14.0)	**0.041**[a]	15 (31.3)
HER-2 (3+)	8 (17.8)	0.9	9 (18.4)
Cyclin D1	28 (56.0)	0.9	28 (56.8)
Cyclin D3	18 (38.3)	0.1	26 (52.0)
Cyclin E	11 (22.0)	0.6	9 (18.0)
Cyclin A	20 (40.0)	0.4	23 (46.9)
Cyclin B1	9 (19.6)	0.3	6 (12.2)
p16	29 (67.4)	0.8	34 (69.4)
p21	18 (36.7)	**0.042**[a]	9 (18.4)
p27	27 (55.1)	0.1	34 (69.4)
Skp2	23 (46.9)	0.7	22 (44.0)
Rb	33 (68.8)	0.1	41 (82.0)
E2F6	17 (34.0)	0.8	17 (34.0)
CHEK2	9 (22.5)	0.6	12 (27.3)
Topo IIα	20 (41.7)	0.8	22 (44.0)
MDM2	7 (14.6)	0.8	8 (16.0)
CK5/6	3 (6.0)	0.1	7 (14.9)
CK8	39 (79.6)	0.8	38 (77.6)
Vimentin	8 (17.0)	0.3	12 (25.5)
Survivin	13 (28.9)	0.5	16 (34.8)
EGFR	6 (14.0)	0.8	7 (14.9)

ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; EGFR, epidermal growth factor receptor.
Unadjusted *P*-value for the comparison between non-*BRCA1/2* and sporadic tumors.
[a]After corrections for multiple comparisons only the association with grade remained significant ($P<0.04$).

immunohistochemical markers, and identified the same five subgroups, with the same pattern of immunohistochemical expression, and similar proportions in each subgroup; 16% HER-2 positive; 15% basal like; 11% normal breast like; 42% luminal A; and 16% luminal B. We validated these results using the Silhouette Width technique (data not shown).

Somatic Inactivation of the *BRCA1* Gene

We observed promoter hypermethylation of *BRCA1* in 21 (42%) of the 50 cases, and these were distributed evenly over the two main groups (Figure 1). There was no evidence of an association between hypermethylation and grade, or any of the immunohistochemical markers studied.

We tested for LOH at the *BRCA1* locus in 19 of the 21 cases with promoter hypermethylation of *BRCA1* (there was no DNA from lymphocytes available for the other 2 cases with promoter hypermethylation), and observed LOH in seven (37%). All seven tumors with double somatic *BRCA1* inactivation were in the high-grade branch and four (57%) of those were in the basal-like group (Figure 1).

Discussion

Immunohistochemical analysis of *BRCA1* and *BRCA2* tumors has previously shown a good correlation between genotype and phenotype,[16,19,20] but there are very few studies on non-*BRCA1/2* tumors.[18,20,27,33] We have previously reported that non-*BRCA1/2* tumors tend to be grade 1–2 and ER and PR positive, have a low proliferative index, and express p53 to a similar extent to *BRCA2* and sporadic tumors, but less than *BRCA1* tumors.[18–20] The present study has confirmed our previous results with new markers related to cell cycle, apoptosis, proliferation, stromal, and epithelial markers. We can say that non-*BRCA1/2* tumors are of lower grade than sporadic tumors, but they are quite similar with respect to the immunohistochemical markers studied (Table 2).

Immunohistochemistry Classification of Familial Non-*BRCA1/2* Tumors

By using a hierarchical unsupervised cluster analysis with 25 markers and grade, we have established a classification of the non-*BRCA1/2* tumors that demonstrates their heterogeneity. Non-*BRCA1/2* tumors can be divided into two main groups primarily according to their ER status and grade. The first group is characterized by higher grade; ER negativity; and the expression of proteins related to proliferation and cell cycle progression, and the second group by low grade; ER positivity; and overexpression of some cyclin–CDK complex inhibitors, antiapoptotic, and luminal (CK8) proteins (Figure 1).

The two main groups of our analysis can be further divided into five subgroups that are consistent with the classification system established by Sorlie *et al*[32] in sporadic breast cancer, using a cDNA array study, as follows: (1) HER-2 positive; (2) basal like; (3) normal breast like, (4) luminal A, and (5) luminal B. In addition, we performed an unsupervised cluster analysis of 50 sporadic tumors using the same immunohistochemical markers used in the non-*BRCA1/2* group, and obtained similar subgroups. Thus, we conclude that non-*BRCA1/2* tumors are heterogeneous and that the immunohistochemical classification is very similar to that found for sporadic tumors using both expression arrays[32] and immunohistochemical markers. We obtained consistent results after excluding families with any ovarian or male breast cancers (data not shown).

Table 3 Immunohistochemical markers that significantly differentiate non-BRCA1/2 tumors with and without somatic BRCA1 inactivation (promoter hypermethylation and LOH of the BRCA1 allele), and comparisons with BRCA1 tumors

	Non-BRCA1/2 tumors without somatic BRCA1 inactivation (n = *41*) *(%)*	P*	*Non-BRCA1/2 tumors with somatic BRCA1 inactivation* (n = *7*) *(%)*	P**	*BRCA1 tumors* (n = *33*) *(%)*	P***
Grade						
1	23 (63.9)		0	0.2	0	
2	8 (22.2)		0		5 (16.1)	
3	5 (13.9)	<0.001	6 (100.0)		26 (83.9)	<0.001
ER						
Negative	14 (34.1)		6 (85.7)	0.4	23 (71.9)	
Positive	27 (65.9)	0.011[a]	1 (14.3)		9 (28.1)	0.001
PR						
Negative	15 (36.6)		7 (100.0)	0.1	26 (78.8)	
Positive	26 (63.4)	0.002	0		7 (21.2)	<0.001
BCL2						
Negative	20 (48.8)		7 (100.0)	0.2	28 (84.8)	
Positive	21 (51.2)	0.012[a]	0		5 (15.2)	0.001
Ki-67						
0–5%	32 (78.0)		1 (14.3)	0.6	9 (27.3)	
6–25%	7 (17.1)		2 (28.6)		11 (33.3)	
>25%	2 (4.9)	<0.001	4 (57.1)		13 (39.4)	<0.001
p53						
Negative	38 (92.7)		3 (42.9)	0.6	17 (51.5)	
Positive	3 (7.3)	0.001	4 (57.1)		16 (48.5)	<0.001
Cyclin E						
Negative	35 (85.4)		2 (28.6)	0.3	15 (46.9)	
Positive	6 (14.6)	0.001	5 (71.4)		16 (53.1)	<0.001
Cyclin A						
Negative	29 (70.7)		0	0.3	4 (12.1)	
Positive	12 (29.3)	<0.001	7 (100.0)		29 (87.9)	<0.001
p27						
Negative	16 (40.0)		6 (85.7)	0.2	21 (63.6)	
Positive	24 (60.0)	0.025[a]	1 (14.3)		12 (36.4)	0.044[a]
Skp2						
Negative	25 (62.5)		0	0.2	5 (15.6)	
Positive	15 (37.5)	0.002	7 (100.0)		27 (84.4)	<0.001
CHEK2						
Negative	26 (83.9)		3 (42.9)	1.0	12 (42.9)	
Positive	5 (16.1)	0.021[a]	4 (57.1)		16 (57.1)	0.001
CK5/6						
Negative	40 (97.6)		5 (71.4)	0.4	19 (57.6)	
Positive	1 (2.4)	0.008[a]	2 (28.6)		14 (42.4)	<0.001
CK8						
Negative	4 (10.0)		5 (71.4)	0.4	18 (54.5)	
Positive	36 (90.0)	<0.001	2 (28.6)		15 (45.5)	<0.001
Vimentin						
Negative	35 (92.1)		2 (28.6)	0.3	15 (46.9)	
Positive	3 (7.9)	<0.001	5 (71.4)		17 (53.1)	<0.001
Survivin						
Negative	29 (80.6)		1 (14.3)	0.1	12 (42.9)	
Positive	7 (19.4)	<0.001	6 (85.7)		16 (57.1)	0.002
EGFR						
Negative	33 (97.1)		2 (28.6)	0.2	15 (53.6)	
Positive	1 (2.9)	<0.001	5 (71.4)		13 (46.4)	<0.001

ER, estrogen receptor; PR, progesterone receptor; EGFR, epidermal growth factor receptor.

[a]No longer significant after correcting for multiple testing.

**P*-value for comparison between non-*BRCA1/2* without and with somatic *BRCA1* inactivation.

***P*-value for comparison between non-*BRCA1/2* with somatic *BRCA1* inactivation and *BRCA1* tumors.

****P*-value for comparison between non-*BRCA1/2* without somatic *BRCA1* inactivation and *BRCA1* tumors.

Somatic Inactivation of the *BRCA1* Gene

We found that 42% of the non-*BRCA1/2* tumors presented promoter hypermethylation of the *BRCA1* gene, a percentage that is higher than in sporadic cases, where it ranges between 11 and 30%.[34,35] However these tumors were evenly distributed across both groups and hypermethylation was not associated with estrogen or progesterone receptor status, or (higher) grade, as has been suggested by some authors.[35,36] Seven (37%) of the non-*BRCA1/2* tumors with promoter hypermethylation also had LOH at, and therefore total inactivation of, *BRCA1*. All seven were all in the high-grade group and the majority showed the basal-like phenotype (Figure 1), the latter being characteristic of *BRCA1* tumors.[37–40] Although the sample size was small, we compared this subgroup with the rest of the non-*BRCA1/2* tumors and found significant differences for the majority of the markers based on unadjusted *P*-values (Table 3). Consistent differences were observed when 33 *BRCA1* tumors were compared with the latter group using previously published data.[18] However, the same comparison between the seven *BRCA1/2* tumors with somatic *BRCA1* inactivation and the 33 *BRCA1* tumors did not reveal significant differences even before correction for multiple testing (Table 3). In addition, six out of the seven tumors with *BRCA1* promoter hypermethylation and LOH showed morphologic characteristics of medullary carcinoma, a subtype associated with *BRCA1* tumors.[41] All these observations suggest that in the majority of cases, a double somatic 'hit' in the *BRCA1* gene is necessary for non-*BRCA1/2* tumors to generate a *BRCA1* phenotype; the first hit would be promoter hypermethylation and the second hit an LOH of the wild-type allele, and both would occur early in tumorigenesis in order to be able to mimic the immunohistochemical profile of *BRCA1* tumors.

Genetic Implications of the New Familial Non-*BRCA1/2* Tumor Classification

The classification by immunohistochemistry of non-*BRCA1/2* tumors into five subgroups confirms that non-*BRCA1/2* tumors constitute a heterogeneous group of tumors, and it supports the hypothesis that the majority of familial non-*BRCA1/2* tumors might be explained by a polygenic model (that is, multiple low-penetrance genes),[15] rather than by a single *BRCAX* gene. That is, our results could represent a practical validation of this hypothesis because we have found that non-*BRCA1/2* tumors have the same immunohistochemical profile as that described in sporadic breast tumors, a type of tumor classically associated with a polygenetic model. This concept does not exclude the existence of some genes that could each explain a small number of families,[13,14] as was recently shown for *CHEK2*.[42,43] In fact, our classification could be very useful in defining more homogenous groups for linkage and other studies designed to identify such genes.

Clinical Implications of the *BRCA1*-like Group

The same mechanisms of somatic inactivation of the *BRCA1* gene that we have described in 14% of our cases was recently observed in a group of sporadic breast tumors,[35] and these tumors were also high grade and ER negative. These data confirm the specific characteristics (high grade and ER negative) of this group of cases that exhibit what has generically been named 'BRCAness', and that both sporadic[44] and familial breast tumors can present as 'BRCA like'. Some experimental studies using demethylating agents are now being conducted on different tumors with good results,[45] and this opens up a new avenue for the treatment of tumors with an allele inactivated by hypermethylation that has to be explored further. On the other hand, the identification of this group of tumors with 'BRCAness' also provides a basis for new therapeutic strategies based on the sensitivity to DNA-damaging agents that *BRCA1* tumors present.[46] DNA repair protein PARP inhibitors and DNA crosslinking agents (cisplatin, mytomycin C, diepoxibutane) seem to affect tumor cells with *BRCA1* mutations by inhibiting the DNA repair of single strand breaks and increasing their non-viability, while leaving normal cells or cells with a functional *BRCA1* allele unaffected.[47] Therefore, *BRCA1* tumors occurring as a result of constitutional or somatic mutations could represent a new group for targeted therapeutic strategies.

Acknowledgement

This work was partially financed by grants from the Ministry of Health and Ministry of Science, FIS04-2224 and SAF03-02497, respectively. EH was financed by the Asociación Española Contra el Cáncer (AECC). We thank the Spanish National Tumor Bank Network for providing samples; Lydia Sánchez, Ana Díez, Mª Jesús Acuña, and Raquel Pajares for their technical assistance; and Jaime Huertas for kind help with CAAT.

Disclosure/conflict of interest

The authors declare that they have no conflict of interest.

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Int. J. Cancer: **120,** 714–717 (2006)

SHORT REPORT

Genomic analysis of the 8p11-12 amplicon in familial breast cancer

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Amplification of 8p11-12 has been recurrently reported in sporadic breast cancer. These studies define a complex molecular structure with a set of minimal amplified regions, and different putative oncogenes that show a strong correlation between amplification and over-expression such as *ZNF703*/*FLJ14299*, *SPFH2*/*C8orf2*, *BRF2* and *RAB11FIP*. However, none of these studies were carried out on familial breast malignancies. We have studied the incidence, molecular features and clinical value of this amplification in familial breast tumors associated with *BRCA1*, *BRCA2* and non-*BRCA1*/*2* gene mutations. We detected 9 out of 80 familial tumors with this amplicon by chromosomal comparative genomic hybridization. Next, we used a high-resolution comparative genomic hybridization array covering the 8p11-12 region to characterize this chromosomal region. This approach allowed us to define 2 cores of common amplification that largely overlap with those reported in sporadic tumors. Our findings confirm the molecular complexity of this chromosomal region and indicate that this genomic event is a common alteration in breast cancer, present not only in sporadic but also in familial tumors. Finally, we found correlation between the 8p11-12 amplification and proliferation (Ki-67) and cyclin E expression, which further proves in familial tumors the poor prognosis association previously reported in sporadic breast cancer.

Key words: 8p11-12 amplification; array CGH; familial breast cancer

The short arm of chromosome 8 is frequently altered in solid and hematological human tumors.[1–4] Amplification at 8p11-12 has been reported in 10–15% of sporadic breast cancer,[3,5,6] although according to recent studies, using high-resolution array comparative genomic hybridization (aCGH) techniques, this frequency may be as high as 25%.[7,8]

Recently, 3 different groups have characterized the 8p11-12 amplification in sporadic breast tumors and breast cancer cell lines using aCGH, to define the region and describe relevant genes that map in this chromosomal location.[7–9] Gelsi-Boyer *et al.*[9] defined 4 minimal amplicons in the 8p11-12 amplified region (Fig. 1). The most telomeric core of amplification (A1) spans 1.27 Mb and comprises the entire minimal region previously described by Garcia *et al.*[8]; this minimal region of common amplification is 1 Mb long and contains several candidate oncogenes (Fig. 1). This narrow region was similarly reported by a third group that suggests breakpoints and complex chromosomal rearrangements within the *NRG1* locus (31.38–32.70 Mb) as likely mechanisms involved in the amplification.[7] The second amplicon (A2), centromeric to A1, has a length of around 800 Kb and contains among other genes *FGFR1*. This gene has been previously proposed as candidate oncogene but its role as target of the 8p12 amplification remains to be established.[3–5,11–16] Two more amplicons, A3 and A4, centromeric to the previous ones and with lengths of 1.25 Mb and 460 Kb, respectively, were also described. Candidate oncogenes at each of these amplicons are listed in Figure 1.

Two regions of recurrent breakpoints in 8p have also been described in breast cancer: a telomeric cluster of breakpoints (BPC1) associated with rearrangements at the *NRG1* locus (31.38–32.70 Mb),[9,10,17] and a centromeric cluster of breakpoints (BPC2) proximal to the *NRG1* and *UNC5D* genes[8–10] (Fig. 1). The telomeric region of 8p distal to these breakpoints is frequently deleted as a result of these breaks.[8–10] This pattern of molecular complexity has been recently reported by us, not only in breast cancer cell lines but also in colon and pancreatic cancer cell lines.[10]

All these data suggest that the 8p11-12 region is prone to present breaks and complex rearrangements, and could therefore participate in oncogenesis *via* inactivation of one or several potential tumor suppressor genes, and/or *via* amplification and overexpression of candidate oncogenes.

We have previously reported 9 cases (11.25%) showing 8p11-12 amplification in a set of 80 familial breast tumors (including 26 *BRCA1*, 18 *BRCA2* and 36 non-*BRCA1*/*2* tumors), using chromosomal comparative genomic hybridization (cCGH).[18] Because familial tumors present different genetic and immunohistochemical (IHC) profiles among themselves and also with respect to sporadic tumors, we decided to investigate the features of this amplicon at the molecular level, to establish whether it is a common event in all breast cancer types.

Patients included in our previous study[18] belonged to families with at least 3 women affected with breast/ovarian cancer and at least 1 of them diagnosed before 50 years, or to families with female breast/ovarian cancer and at least with 1 case of male breast cancer. All cases were studied for mutations in *BRCA1* and *BRCA2* genes and for large rearrangements alterations using standard procedures.[19] Those that were negative for this mutational screening were considered as non-*BRCA1*/2 patients. We extracted DNA from paraffin-embedded tissue from the 9 familial

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0020-7136/suppmat.

JDB is a Cancer Research UK Senior Clinical Research Fellow, Cancer Research, UK.

Grant sponsor: Spanish Ministries of Health and Science; Grant numbers: FIS04-2224, SAF03-02497; LM Grant sponsor: Spanish Ministry of Science; Grant number: AP-2004-0448; EH Grant sponsor: Spanish Association Against Cancer (AECC); Grant sponsor: Cancer Research, UK.

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Received 17 July 2006; Accepted 30 August 2006
DOI 10.1002/ijc.22354
Published online 9 November 2006 in Wiley InterScience (www.interscience.wiley.com).

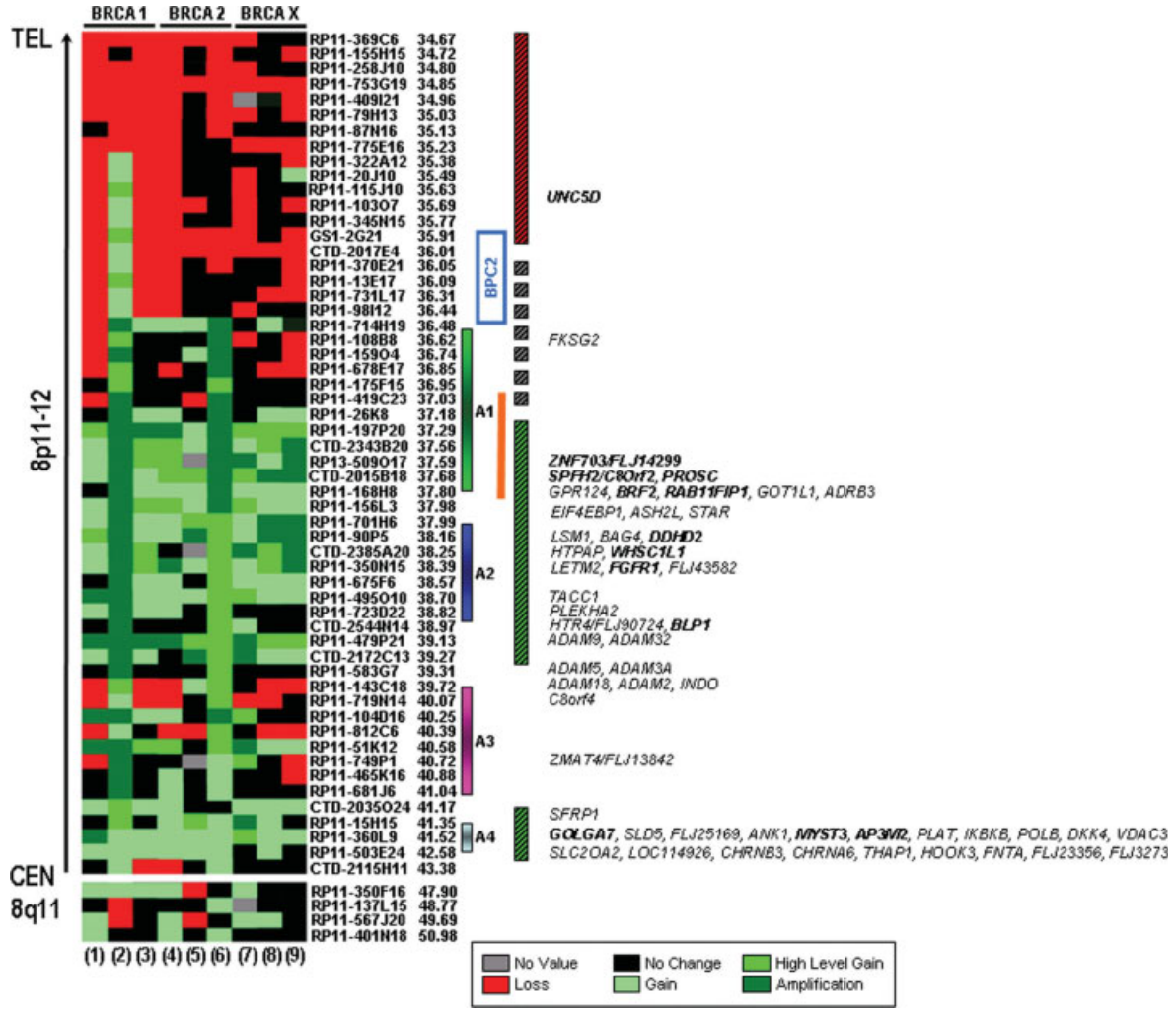

FIGURE 1 – Array-CGH profile across the 8p11-12 region. The 9 samples are represented in columns: 3 BRCA1 (1–3), 3 BRCA2 (4–6) and 3 BRCAX (7–9). Each row represents a clone on the array. Clones have been ordered by genome position according to the NCBI Build 35 from the most distal ones (top) to the most centromeric ones (bottom). The midpoint position (Mb) of each clone is indicated beside the clone name, as well as the genes mapping in the region. Colors represent discrete values according to the aCGH ratio: red indicates loss (ratios <0.8), black implies no change (0.8–1.2) and a green scale represents distinct levels of gains. Light green represents simple gain (1.2–1.5), midtone green is high-level gain (1.5–1.75) and dark green represents BAC amplification (>1.75). Grey cells correspond to data rejected after quality tests for signal intensity and replicate reproducibility. Genes in bold font present a good correlation between amplification and expression levels according to previous studies. Left colored thick bars represent the 4 cores of amplification described by Gelsi-Boyer *et al*. (A1, A2, A3, A4).[9] The orange bar defines the 1-Mb minimal amplicon reported by us in a previous study.[8] The blue box represents the cluster of breakpoints proximal to the *UNC5D* gene reported by Gelsi-Boyer *et al*. (BPC2).[9,10] Finally, the further right colored striped bars represent the region of loss distal to breakpoints (red box), the area where breaks mainly occurred (discontinuous grey boxes) and the 2 regions of gain/amplification (green boxes) we describe.

breast tumor samples in our collection (3 BRCA1, 3 BRCA2 and 3 non-BRCA1/2) that displayed amplification at the 8p11-12 region. We used a xylene treatment and sodium thiocyanate incubation before proteinase K digestion and phenol chloroform extraction. We then hybridized these cases onto a new version of a previously used in-house BAC-array.[8,10,17] Briefly, this platform comprised BACs ~10 Mb apart across the whole genome; 1.5 Mb apart over chromosome 8; and at near-tiling-path density over 8p11-12. Interestingly, its resolution at 8p11-12 was further improved by adding 30 more clones to fill in existing gaps at the region. A total number of 91 BACs (*versus* 61 in the previous set) spans over 9.5 Mb at 8p11-12 between positions 31.03 Mb (RP11-473A17) and 43.38 Mb (CTD-2115H11). Name, position, size and accession number for these clones are available in Supplementary Table I. DNA labeling and hybridization as well as image acquisition and data analysis were done as previously published.[8] Clinical and IHC data of the 80 cases studied by cCGH were collected from earlier studies by our group.[20,21]

The genomic characterization of the 8p11-12 region in the 9 cases with amplification by means of hybridization on the high-resolution aCGH platform is summarized in Figure 1. The majority of the cases did not show any rearrangements at the previously described cluster of breakpoints BPC1, which has been associated with the *NRG1* locus. In contrast, most of breakages occurred roughly between BACs CTD-2017E4 (36.01 Mb) and RP11-26K8 (37.18 Mb). Genomic losses telomeric to the breakpoints and gain/amplification centromeric to the breakpoints were observed, reproducing the genomic imbalances already defined at this chromosomal region.[8,9] This region of breakpoints overlaps with the previously described cluster of breakpoints centromeric to the *UNC5D* gene, BPC2 (Fig. 1).[9,10] These findings highlight this area as prone to develop breaks in different types of tumors, including

TABLE I – CORRELATIONS BETWEEN 8p11-12 AMPLIFICATION AND IMMUNOHISTOCHEMICAL AND CLINICAL FEATURES

	No amplification, *n* (%)	8p11-12 amplification,[1] *n* (%)	*p**
Age (years)			
<44	25 (45.5)	5 (55.6)	NS
≥44	30 (54.5)	4 (44.4)	
SBR grade			
1	17 (28.3)	1 (11.1)	NS
2	17 (28.3)	2 (22.2)	
3	26 (43.3)	6 (66.7)	
Estrogen receptor			
<10	24 (35.8)	4 (44.4)	NS
≥10	43 (64.2)	5 (55.6)	
Progesterone receptor			
<10	32 (47.8)	5 (55.6)	NS
≥10	35 (52.2)	4 (44.4)	
p53			
<25	51 (77.3)	7 (77.8)	NS
≥25	15 (22.7)	2 (22.2)	
Ki-67			
<20	49 (74.2)	3 (33.3)	0.013
≥20	17 (25.8)	6 (66.7)	
Cyclin D1			
<25	32 (51.6)	4 (50.0)	NS
≥25	30 (48.4)	4 (50.0)	
Cyclin D3			
<10	34 (56.7)	5 (71.4)	NS
≥10	26 (43.3)	2 (28.6)	
Cyclin E			
<10	46 (74.2)	3 (37.5)	0.033
≥10	16 (25.8)	5 (62.5)	
Cyclin A			
<10	23 (37.7)	1 (12.5)	NS
≥10	38 (62.3)	7 (87.5)	
Cyclin B1			
<10	41 (68.3)	3 (42.9)	NS
≥10	19 (31.7)	4 (57.1)	
p16			
<50	22 (40.0)	3 (37.5)	NS
≥50	33 (60.0)	5 (62.5)	
p21			
<10	35 (57.4)	2 (25.0)	NS
≥10	26 (42.6)	6 (75.0)	
p27			
<50	26 (42.6)	4 (50.0)	NS
≥50	35 (57.4)	4 (50.0)	

[1]Cases whose 8p11-12 amplification was defined by cCGH and studied in the present work.

*p-value defined by Pearson's χ^2 test. NS, non significant.

familial breast cancer. Regarding amplified regions, all cases showed distinct levels of gains that were grouped in 2 main subregions (Fig. 1). The first one has a length of 2.13 Mb, and comprises the proximal half of A1, the whole of A2, and a small region of around 100 Kb between A2 and A3. This genomic area contains genes such as *ZNF703/FLJ14299, SPFH2/C8orf2*, *PROSC, DDHD2*, *WHSC1L1* and *FGFR1*, all of them previously reported as genes of interest for further functional analysis because of their amplification-over-expression correlation.[7–9] It is important to note that this chromosomal region includes the minimal 1 Mb amplicon already defined by us,[8] supporting the candidate role of the genes located there. The second subregion encompasses the A4 core of amplification, has a length of 1.41 Mb, shows a lower level of gain and contains other genes already described as relevant such as *GOLGA7*, *MYST3* and *AP3M2*.[1,9] Interestingly, the majority of cases in our sample set did not present amplification at the A3 region. This region was amplified in only 2 cases (cases 2 and 6) that presented large and continuous amplifications from about positions 35 and 36 Mb up to the centromere (Fig. 1). No differences in the pattern and distribution of the amplifications were observed among the 3 types of familial breast tumors (BRCA1, BRCA2 and non-BRCA1/2).

Amplification of 8p11-12 has been associated with high proliferation (high histological grade and Ki-67 expression)[9] and an adverse effect on survival in breast cancer.[7,9] To evaluate these clinical associations in our series, we compared different IHC markers and clinical variables between the 9 tumors with this amplification and those cases without this genomic event (68 samples) (Table I). We found that tumors with 8p11-12 amplification had significantly higher Ki-67 and cyclin E expression ($p = 0.013$ and 0.033, respectively). Moreover, we observed a trend to present high grade, high expression of other cell cycle markers (such as cyclin A or B1) and an early age of onset in tumors with the amplification. These differences were not statistically significant, probably due to the low number of cases (Table I). These clinical and IHC features have been largely associated with tumor progression, proliferation and poor patient prognosis.[22–27] Therefore our data indicate that this amplified region would present clinical prognostic value in familial breast cancer, as it has been shown for sporadic breast tumors. Interestingly, the region A2 described as the amplification core associated with the most aggressive tumor behavior[9] was amplified in all of our cases.

In summary, to our knowledge, this is the first time that the 8p11-12 amplification has been reported and analyzed in detail in familial breast cancer. We have defined 2 common regions of amplification that greatly overlap with the minimal regions of amplifications previously described in sporadic tumors. We also found a cluster of breakpoints centromeric to the *UNC5D* gene, similarly to what has been reported for sporadic neoplasms. Therefore our findings in a selected group of familial tumors confirm the molecular complexity of the 8p11-12 chromosomal region and suggest that these alterations, and probably some gene(s) mapping in these regions, are common in breast cancer pathogenesis, independent of the tumor type. Finally, we have found that the presence of this amplification is associated with high proliferation (Ki67) and cyclin E expression, which further supports the clinical value of this aberrations found in sporadic breast cancer.

Acknowledgements

We thank the Spanish National Tumor Bank Network, the Familial Cancer Unit, the Immunohistological Unit and the Molecular Cytogenetics Group of the Spanish National Cancer Center for their technical support.

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Estrogen Receptor Status Could Modulate the Genomic Pattern in Familial and Sporadic Breast Cancer

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Abstract

Purpose: Familial breast cancer represents 5% to 10% of all breast tumors. Mutations in the two known major breast cancer susceptibility genes, *BRCA1* and *BRCA2*, account for a minority of familial breast cancer, whereas families without mutations in these genes (BRCAX group) account for 70% of familial breast cancer cases.

Experimental Design: To better characterize and define the genomic differences between the three classes of familial tumors and sporadic malignancies, we have analyzed 19 BRCA1, 24 BRCA2, and 31 BRCAX samples from familial breast cancer patients and 19 sporadic breast tumors using a 1-Mb resolution bacterial artificial chromosome array-based comparative genomic hybridization.

Results: We found that BRCA1/2 tumors showed a higher genomic instability than BRCAX and sporadic cancers. There were common genomic alterations present in all breast cancer groups, such as gains of 1q and 16p or losses of 8ptel-p12 and 16q. We found that the presence/absence of the estrogen receptor (ER) may play a crucial role in driving tumor development through distinct genomic pathways independently of the tumor type (sporadic or familial) and mutation status (BRCA1 or BRCA2). ER^- tumors presented higher genomic instability and different altered regions than ER^+ ones.

Conclusions: According to our results, the BRCA gene mutation status (mainly BRCA1) would contribute to the genomic profile of abnormalities by increasing or modulating the genome instability.

Two major genes associated with susceptibility to hereditary breast cancer have been identified to date: *BRCA1* and *BRCA2* (1, 2). Inheritance of a mutation in these genes confers an increased lifetime risk of breast cancer (60-85%) and ovarian cancers (15-40%; ref. 3), although these genes only explain ~25% of breast cancers within high-risk families (4, 5). Some groups have tried to find putative BRCAX gene(s) using linkage analysis to explain the genetic background of some of the remaining high-risk families but without conclusive results (6–9).

A large amount of data have been presented showing that breast tumors from patients with germ-line mutations in the *BRCA1* and *BRCA2* genes present morphologic and genetic differences and also differ from BRCAX tumors and sporadic breast cancer cases (see refs. 10, 11 for review). These data suggest that hereditary mutations in *BRCA1* and *BRCA2* lead to breast cancer development through different signaling pathways. One feature of solid tumors, and thus of breast cancer, is genomic instability, which involves chromosomal changes, such as DNA gains or losses. To understand these changes, many groups have pursued tumor genome profiling of different classes of breast cancer. By chromosomal comparative genomic hybridization (cCGH; ref. 12), *BRCA1*-associated tumors are characterized by a high frequency of losses of 5q, 4q, 4p, 2q, and 12q; whereas *BRCA2*-associated tumors present frequent losses of 13q and 6q and gains of 17q22-q24 (13). In addition, and because of the recent development of classifiers based on specific DNA copy number alterations of each tumor class (14–16), cCGH profiling has been proposed as a potential diagnostic tool.

More recently, array-based CGH (aCGH) has become widely used, providing higher resolution and flexibility than cCGH (see refs. 17–19 for review). The single analysis of familial breast cancer using aCGH published to date has confirmed

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Received 3/28/07; revised 7/5/07; accepted 9/14/07.

Grant support: Spanish Ministry of Education and Science grant FPU AP-2004-0448 (L. Melchor), ICRETT/05/063 and European Molecular Biology Organization Short-Term Fellowship ASTF 162-05, Spanish Ministry of Education and Science projects SAF-03-02497 and SAF-06-06149, and Breast Cancer Research Foundation (K.L. Nathanson).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-07-0711

some previous findings, such as the higher genomic instability and specific losses of 4p, 4q, and 5q observed in *BRCA1*-associated tumors and frequent gains of 17q24 found in breast cancers associated with *BRCA2* mutations (20). The authors also described a set of chromosomal regions that correctly discriminated among BRCA1, BRCA2, and sporadic breast tumors. However, BRCAX cases were not included in this study, and the results need to be confirmed in larger and independent series.

In the present study, we aimed to, first, establish the genomic profile of sporadic and BRCA1, BRCA2, and BRCAX familial breast cancers using a 1-Mb resolution bacterial artificial chromosome (BAC) array platform and, second, identify regions commonly altered in all groups and regions that are class specific. Finally, because the array platform we used was the same as the one implemented by Jönsson et al., we tried to validate the aberrant regions they proposed in our tumor sample set.

Materials and Methods

Patients and tumor samples. We collected formalin-fixed, paraffin-embedded (FFPE) breast tumor tissues from 74 patients. These patients were selected from families with at least three women affected with breast/ovarian cancer and at least one of them diagnosed before 50 years of age or from families with female breast/ovarian cancer and at least one case of male breast cancer. All cases were studied for mutations and large rearrangements in the BRCA genes using standard procedures (4, 21). Nineteen cases had mutations in the *BRCA1* gene, 24 patients presented mutations in the *BRCA2* gene, and 31 cases were negative for germ-line mutations in the BRCA genes and therefore denoted as BRCAX tumors. Eight of the breast cancers with *BRCA2* mutations were provided by the Breast Cancer Genetics Team, Institute of Cancer Research (Sutton, Surrey, United Kingdom). We also included a set of snap-frozen sporadic breast tumors from 19 unselected patients without a family history of breast cancer. All data on sporadic samples were provided by the University of Pennsylvania and included in a previous study (22). BRCA gene mutation status, age at diagnosis, type of carcinoma, histologic grade, and estrogen receptor (ER) status are provided in Supplementary Table S1. ER status was considered as positive when the percentage of stained cells was $\geq$10% in an immunohistochemistry analysis, as previously reported (23, 24).

DNA extraction. Genomic DNA from frozen tissue sections was extracted using proteinase K digestion followed by phenol/chloroform purification. DNA from FFPE tumors was isolated according to the S. DeVries/F. Waldman protocol[6] with minor modifications. Briefly, two 30-μm sections were obtained from FFPE tumors, treated with xylene, incubated in glycine-Tris-EDTA [100 mmol/L glycine, 10 mmol/L Tris (pH 8.0), 1 mmol/L EDTA] and sodium sulfocyanate (1 mol/L), and finally digested with proteinase K and purified with phenol chloroform. All sections were previously examined and dissected with a scalpel to ensure at least 70% content of tumor cells.

BAC array platform. CGH was done onto the "1-Mb array" platform developed at the University of Pennsylvania (25) and previously used in similar studies (20, 22). Briefly, the platform is composed of a set of 4,134 publicly available human BAC clones spaced at ~1-Mb resolution across the genome. BAC DNA was amplified using oligonucleotide-primed PCR primers. At least two replicates of each BAC clone were printed on each slide using a Lucidea Array Spotter (Amersham Biosciences) and a spotting solution of 50% DMSO (25).

Array comparative genomic hybridization. For hybridization, 1 μg of test DNA and 1 μg of sex-matched pooled normal human DNA (obtained from a set of 10 healthy female or male volunteers) were labeled with either Cy3-dCTP or Cy5-dCTP by random priming (BioPrime Labeling kit, Invitrogen). The differentially labeled DNA samples were pooled, mixed with 100 μg of human COT-1 DNA, dried down, and rehydrated in 50 μL of a formamide-based buffer (25). After denaturation (10 min at 75°C) and preannealing (30 min at 37°C), hybridization was allowed for 48 to 72 h at 37°C in a moist chamber on a slowly rocking platform followed by a series of posthybridization washes: 2× SSC and 0.1% SDS (15 min, room temperature), 2× SSC and 50% formamide, pH 7.0 (15 min, 45°C), 2× SSC and 0.1% SDS (30 min, 45°C), and 0.2× SSC (15 min, room temperature). Finally, arrays were scanned on a GenePix 4000B dual scanner (Axon Instruments). Both test and reference DNA were labeled with the opposite dye in a separate experiment ("dye swap") to account for differences in dye incorporation and provide additional data points for analysis.

Data analysis. Fluorescence data from hybridization images were processed and analyzed with GenePix Pro 5.0 (Axon Instruments) to obtain the $\log_2$ ratios (tumor/reference) of each slide. aCGH normalization was done using the DNMAD application (26), which also allowed us to merge and filter replicate clones on the same slide and in the dye-swap experiment. We filtered out inconsistent replicates (those with a $\log_2$ ratio distance to the median $\log_2$ ratio of the replicates >0.3) and those clones that did not have available data in >70% of the cases (65 of 93 samples).

Finally, we established categorized copy number values using the binary segmentation algorithm implemented in the InSilico CGH software (0, 1, or -1 indicating no change, gain, or loss, respectively; ref. 27). We defined genomic regions as a group of at least two consecutive clones showing the same categorized copy number value. High-level DNA amplifications were considered when segmentation level was four times the segmentation gain level.

For visualization purposes, we used CGHAnalyzer (25, 28) and CGHExplorer (29) softwares.

Standardization of an artifactual-copy number variation pattern. We observed a recurrent genomic pattern of artifactual aberrations in our breast cancer sample set (see Results), which was previously reported in normal samples analyzed by cCGH (30) and recently also observed by aCGH and named artifactual-copy number variation (Ar-CNV).[7] Briefly, this artifactual pattern generates abnormal ratios in certain chromosomal regions, such as 1p36, 2q37, 4p16, 6p21, 9q34, 11q13, and 12q13 (Fig. 1A), which can be erroneously interpreted as gains in the analysis. We also obtained this Ar-CNV pattern in a set of normal FFPE tissue DNA versus normal control DNA hybridizations. Kirchhoff et al. (30) previously described a nonrandom pattern of deviations in normal samples by cCGH and, subsequently, applied a standardization approach to increase the specificity and the sensitivity of the technique, obtaining a dramatic decrease in false-positive results. Therefore, to diminish the effect of the Ar-CNV in our set, a standardization approach was applied to every sample showing this artifactual pattern. The standardization was done by subtracting from each clone $\log_2$ ratio the median value for that clone in the set of normal FFPE tissue DNA versus normal control DNA hybridizations that also presented the Ar-CNV pattern. After the standardization, cases were analyzed again and the aCGH profiles did not show the artifactual pattern anymore (Fig. 1B). Log_2 ratios of each sample are shown in Supplementary Table S2.

Fluorescence* in situ *hybridization studies. To verify the standardization approach, we did a fluorescence *in situ* hybridization (FISH)

[6] Protocol available from http://cc.ucsf.edu/people/waldman/Protocols/paraffin.html.

[7] D. Blesa et al. Detection of a pattern of artifactual copy number variations that can induce to overestimate changes on genome profiling analysis, submitted for publication.

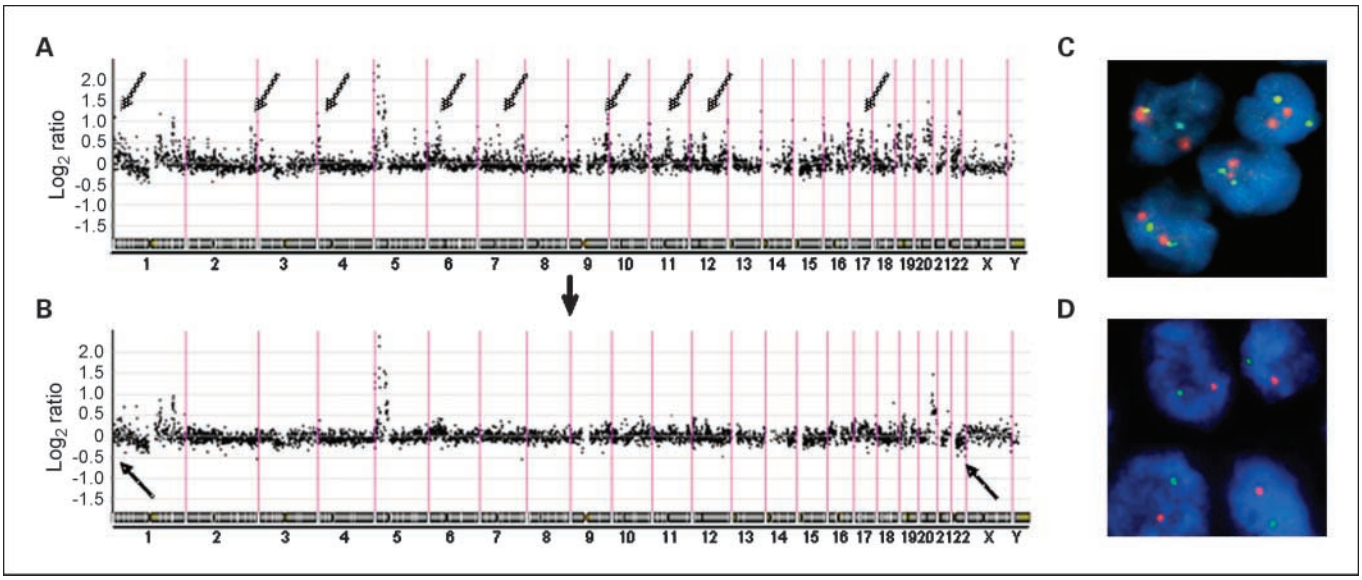

Fig. 1. Standardization of the Ar-CNV pattern. *A,* aCGH profile of a familial breast tumor sample that shows artifactual aberrations in the regions indicated by the arrows. *B,* aCGH profile of the same case after standardization. Aberrations at 1q, 5p, or 20q are considered gains because they are preserved after standardization. Arrows below the tumor profile indicate regions further validated by FISH analysis. *C,* FISH study using the 1p36 probe, showing two green signals (1p36 loci) and two red signals (centromeres). These data support the standardized aCGH pattern and not the nonstandardized one in which the gain was artifactual. *D,* FISH experiment with the chromosome 22 probe clearly shows a monosomy (one green and one red signal), supporting again the loss observed in the standardized profile.

analysis on tissue microarrays that contained the FFPE samples; their characteristics have been previously published (23, 24). We checked the copy number status of two different locations affected by Ar-CNV: 1p36 and 22q11-q12. The 1p36 probe was made of three BAC clones from the distal p-arm region of chromosome 1 (RP11-82D16, RP4-713A8, and RP4-740C4, located at 2.07, 2.25, and 2.30 Mb from the 1p telomere, respectively). These BACs were all labeled with dUTP-SpectrumGreen (Vysis, Inc.). The 1p36 probe also included a chromosome 1 centromeric probe "CEP1α Satellite DNA Spectrum-Orange" from Vysis. The 22q11-q12 probe consisted of two clones mapping at 22q11.21 (RP11-316L10 and RP11-330P17) labeled with dUTP-SpectrumGreen and three clones located on 22q12.2 (RP1-76B20, RP1-15I23, and RP3-394A18) labeled with dUTP-Spectrum-Orange. FISH analysis was done according to Vysis' instructions, with slight modifications. An average of 110 (50-200) well-defined nuclei was analyzed and the number of single copy gene and centromeric signals was scored.

Statistical analysis of aCGH data. We used a nonparametric Mann-Whitney *U* test to compare the mean number of genomic alterations among the four patient groups. The Statistical Package for the Social Sciences for Windows statistical software (SPSS, Inc.) was used for these comparisons. For the analysis of differences in the aberration frequency of specific chromosomal regions, we used the Stat POMELO tool[8] (31), applied Fisher's exact test, and adjusted *P* values for multiple testing using the false discovery rate approach (a *P* value of <0.05 was considered significant). Finally, hierarchical unsupervised clustering was done using correlation methods included in the Cluster software (32).

Results

Standardization procedure to diminish the effect of the Ar-CNV pattern. We analyzed tumor DNA from a total of 93 breast tumors (19 BRCA1, 24 BRCA2, 31 BRCAX, and 19 sporadic samples) using aCGH. We found a specific pattern of aberrations that was present in a high percentage of cases (78%). These genomic aberrations were coincident with the so-called Ar-CNV, described in a recent aCGH technical report. In this article, an aCGH pattern of specific Ar-CNV, observed in normal samples and distinct tumor types by using different array platforms and reproduced in several laboratories, is described.[7] We applied a standardization approach similar to the one used by Kirchhoff et al. (30) in their original cCGH studies to avoid an analogous issue (see Materials and Methods). An example of our standardization approach is shown in Fig. 1A and B. Some of the regions affected by Ar-CNV were further validated by FISH analysis (Fig. 1C and D; Supplementary Table S3). A closer correlation aCGH-FISH data was clearly found after standardization, showing a dramatic increase in confirmed aberrations (9% before to 82% after standardization). This analysis confirmed the utility of this correction.

Overall genomic changes in breast tumor classes. We determined the genome instability according to the number of CNVs (measured as the total number of gains, losses, and numerical aberrations) present in a tumor. *BRCA1*-associated tumors had the most unstable genome with a total of 28.0 ± 2.9 CNV, *BRCA2*-related tumors had 19.8 ± 2.3 CNV, BRCAX tumors showed 15.3 ± 1.9 CNV, whereas sporadic tumors presented 18.7 ± 1.9 CNV. These differences were statistically significant ($P < 0.05$, Mann-Whitney *U* test) when *BRCA1*-associated tumors were compared with the other tumor types (Supplementary Fig. S1A). No significant differences were found when a comparison between different types of recurrent mutations was made (data not shown).

Frequencies of genomic alterations. The frequency and distribution of genomic gains and losses of each group is shown in Fig. 2. Four genomic regions were altered in >40% of cases in all tumor groups: gains of 1q and 16p13.3 and losses of 8ptel-p12 and 16q. No specific aberrations were associated significantly with an exclusive breast tumor class. However, there were

[8] Statistics software available from http://pomelo.bioinfo.cnio.es.

some statistically significant differences in the pairwise comparisons (Table 1). Given that BRCAX tumors showed the lowest overall alteration frequency, many regions in BRCAX tumors were significantly different when compared with the other tumor classes (Table 1). Differences in the alteration frequencies in chromosome X were not considered because the reference for the sporadic breast cancer set was not sex matched.

There were aberrant regions at a high frequency (>50%) in each tumor class (Fig. 2). In summary, losses of 4q32.3-qtel, 5q, 13q, and 18q were more frequent in *BRCA1*-associated tumors. *BRCA2*-associated tumors presented recurrent gains of 8q12.3-qtel and losses of 11q23.1-qtel, 13q12.3-q21.33, and chromosome 22. Sporadic breast cancers had frequent gains of 8q13.1-qtel and losses of 4q24-qtel and 13cen-q31.3. Finally, BRCAX tumors did not show any highly recurrent aberration in addition to the commonly altered regions.

About high-level DNA amplifications, 8q24.11 and 8q24.12-q24.21 were the only two regions with a frequency >15% in all groups. No significant differences in high-level DNA amplification frequencies were found across tumor types. However, different tumor classes showed frequent high-level amplification (>15%) at distinct locations, such as 8p, 8q, 10p, 11q13, 12p13, and 13q34 (Table 1), pointing at subtle differences in the amplification targets of each tumor class. We looked in detail at specific regions of amplification that cover some known oncogenes, such as 8q24.21 (*c-MYC*) and 17q12 (*ERBB2*). *c-MYC* is amplified in all groups (37% BRCA1, 33% BRCA2, 26% sporadic, and 13% BRCAX). *ERBB2* was only amplified in 12.9% of BRCAX and 5.3% of sporadic cancers. Noteworthy, no high-level amplification of this gene was observed in *BRCA1*- and *BRCA2*-associated tumors.

Testing of the previously reported discriminative regions. To test the value of the discriminative regions described by Jönsson et al. (20), we used a hierarchical unsupervised clustering using the same discriminative chromosomal regions. We only included the mutation-positive BRCA carriers and sporadic breast cancer cases because these were the tumor classes previously used to describe these regions. We could distinguish two subgroups: one mainly composed of *BRCA1*-associated tumors (orange cluster) and other one mainly composed of *BRCA2*-associated and sporadic tumors (green cluster; Fig. 3A). However, we found BRCA2 (4) and sporadic (6) outlier tumors located in the BRCA1 subgroup and some BRCA1 samples (7) in the other branch. Jönsson et al. also found some outliers in their clustering. The authors suggested that ER status and grade might explain those outliers. When looking in detail at the ER status in our tumor series, we found that more than half the outliers in the BRCA1 branch (two BRCA2 and five sporadic tumors) were ER^-. Similarly, six of the seven BRCA1 outliers in the BRCA2 branch were ER^+ (Fig. 3A). This might indicate that these regions are mainly differentiating positive and negative ER tumors rather than BRCA mutation status.

Because Jönsson et al. only included in their study ER^- *BRCA1*-associated, ER^+ *BRCA2*-associated, and a mixture of $ER^{-/+}$ sporadic tumors, and we had a mixture of $ER^{-/+}$ in all tumor groups, we removed from our series all those cases that did not match the features of the series studied by Jönsson et al. (ER^+ *BRCA1*-associated and ER^- *BRCA2*-associated tumors). Without these tumors, we obtained a different cluster. The clustering analysis discriminated two groups, one of them (orange) mostly composed of ER^- tumors (17 ER^- and 2 ER^+ tumors) and a second branch (green) composed mainly of ER^+ tumors (22 ER^+ and 3 ER^- tumors; Fig. 3B). This time, the number of BRCA class outliers was reduced from 11 outliers (7 BRCA1 and 4 BRCA2; Fig. 3A) to 3 outliers (1 BRCA1 and 2 BRCA2; Fig. 3B). This cluster resembled the results obtained by Jönsson et al. These findings may highlight the importance of ER status in tumor development.

ER profiling of breast cancer. To further test the role of the ER, we divided all cases according to ER status, accounted for CNV in each group, and created genomic alteration frequency plots. The higher genomic instability in the ER^- tumors was remarkable, showing significant differences ($P < 0.05$) in CNV compared with the ER^+ tumor group (Supplementary Fig. S1B). We also saw this difference when we subdivided each tumor class according to ER status (Supplementary Fig. S1C).

Frequency plots of genomic alterations in the tumor groups according to the ER status are shown in Fig. 4A. Both groups of

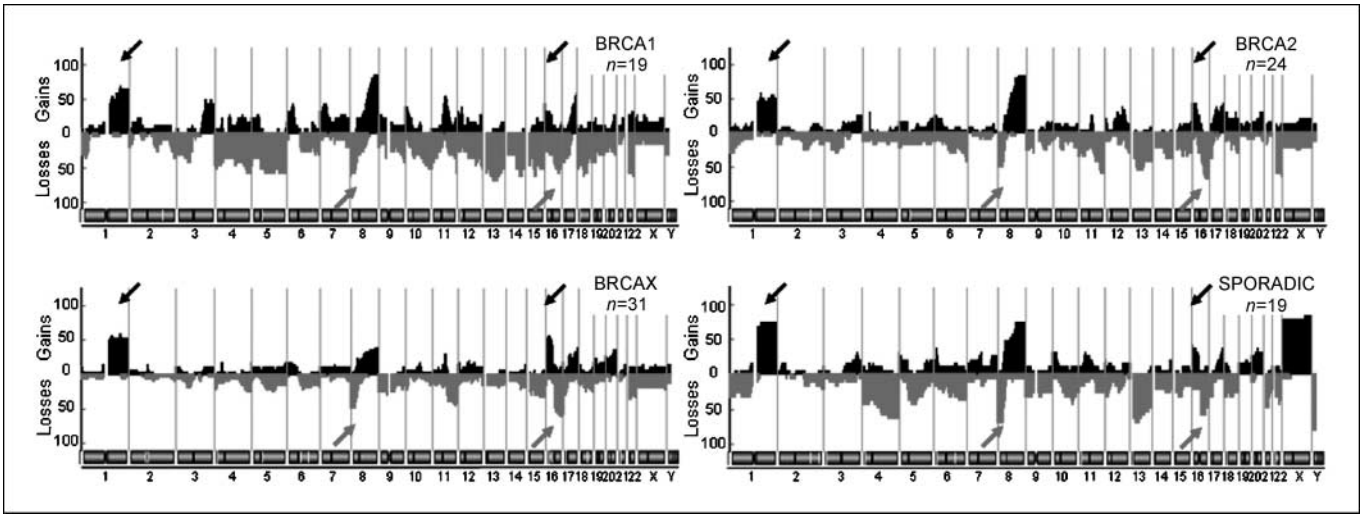

Fig. 2. Overall genomic aberration frequencies in BRCA1, BRCA2, BRCAX, and sporadic breast cancer samples. Black, frequency of gains; gray, frequency of losses. Arrows at chromosomes 1, 8, and 16 highlight the regions commonly altered in >40% of all breast cancer groups. Black arrows, common gains; gray arrows, common losses.

Table 1. Significant genomic aberrations (adjusted $P < 0.05$) and their frequencies among distinct breast tumor groups and recurrent high-level DNA amplifications

Chromosomal region	Frequencies of alteration (%)				Two-sided adjusted Fisher's exact *P*					
	BRCA1	BRCA2	BRCAX	Sporadic	B1vsB2	B1vsBX	B1vsS	B2vsBX	B2vsS	BXvsS
Gain										
8q22.1	**58**	**79**	32	**74**				*0.023*		
8q23.1-8q23.3	**74**	**83**	35	**74**				*0.010*		
8q23.3-8q24.13	**79**	**83**	35	**74**				*0.010*		
8q24.13-8qtel	**84**	**83**	39	**74**		0.077		*0.025*		
11q14.1	47	0	10	10	*0.005*					
Loss										
4q23	32	12	6	53						*0.020*
4q24	37	12	6	**58**						*0.005*
4q25	47	12	6	**58**		0.074				*0.005*
4q26-4q28.1	42	12	6	**58**						*0.005*
4q28.2	42	16	9	**58**						*0.031*
4q28.3	42	16	10	**63**						*0.022*
4q31.1	42	8	13	**63**					*0.009*	*0.022*
4q31.21	37	8	16	**63**					*0.009*	
4q32.1-4q32.2	**47**	12	23	63					*0.044*	
4q32.3	**52**	12	23	63					*0.044*	
4q33-4q34.1	**58**	12	26	63					*0.044*	
4q34.2-4q35.1	**58**	12	23	63					*0.044*	
4q35.1-4q35.2	**52**	12	26	63					*0.044*	
13q12.3	47	**50**	16	**68**						**0.013**
13q13.1-13q13.3	**53**	**50**	16	**68**						**0.013**
13q13.3-13q14.11	**58**	**54**	16	**68**						**0.013**
13q14.11-13q14.3	**63**	**54**	16	**68**		0.097				**0.013**
13q14.3-13q21.33	**68**	**54**	16	**58**		*0.019*				
13q22.1-13q22.2	**68**	42	16	**58**		*0.019*				
13q22.2-13q22.3	**68**	42	19	**58**		*0.048*				
13q31.1	**63**	42	19	**53**		*0.026*				
21q21.3	11	0	3	42					*0.025*	*0.046*
High-level DNA amplifications										
8p12-8p11.23	11	8	6	**16**						
8p11.22-8pcen	11	4	3	**16**						
8q22.1-8q22.3	5	**17**	10	**16**						
8q23.1-8q23.3	11	**21**	10	**21**						
8q24.11	**32**	**37**	**16**	**21**						
8q24.12-8q24.21	**37**	**37**	**16**	**21**						
8q24.21-8q24.22	**37**	**29**	13	**26**						
8q24.23-8qtel	**21**	**25**	10	**26**						
10ptel-10p15.3	0	0	0	**16**						
10p15.2	11	0	0	**16**						
10p15.1	0	0	0	**16**						
10p14	5	0	0	**16**						
11q13.3-11q13.4	11	12	**16**	0						
12p13.32-12p13.31	**16**	0	0	5						
13q34-13qtel	**16**	4	0	11						

NOTE: Frequency aberrations >50% are in bold. Entries in italics show $P < 0.05$. In addition, those high-level DNA amplifications with a frequency >15% (in bold) in at least one tumor group are shown.

tumors have common high frequent gains of 1q and 8q22.1-qtel and losses of 8ptel-p12 and 16q, which are similar to those described in all breast tumor classes. However, a set of genomic aberrations, such as -3p25.3-p21.3, -3p21.1-p14.2, +3q25.1-q26.1, -4q31.2-qtel, -5q, +10p14-p12.3, -12q14-q15, and -12q23.1-q23.31, presented significantly higher frequencies in ER^- tumors (Fig. 4B). It is noteworthy that 6q25.1 (the locus of the *ESR* gene) is more frequently deleted in ER^+ than in ER^- tumors.

In addition, ER^- tumors presented a trend toward having more high-level DNA amplifications than ER^+ tumors. There were also slight differences in the amplification target sites. For example, 8q24.21 (*c-MYC*) was amplified in 32% of the ER^- tumors and only in 20% of the ER^+ tumors. Moreover, 17q12 (*ERBB2*) was exclusively amplified in ER^- tumors (5%), whereas amplification at 20q13.12-q13.33 was only found in ER^+ tumors (8%). However, the only region with an amplification rate that was significantly different (adjusted *P* value < 0.05) comparing both groups was 13q34, which was only present in ER^- tumors (16%).

Discussion

The present aCGH-based study characterizes in detail the patterns of genomic alteration of the familial breast tumor classes (BRCA1, BRCA2, and BRCAX) and of sporadic breast

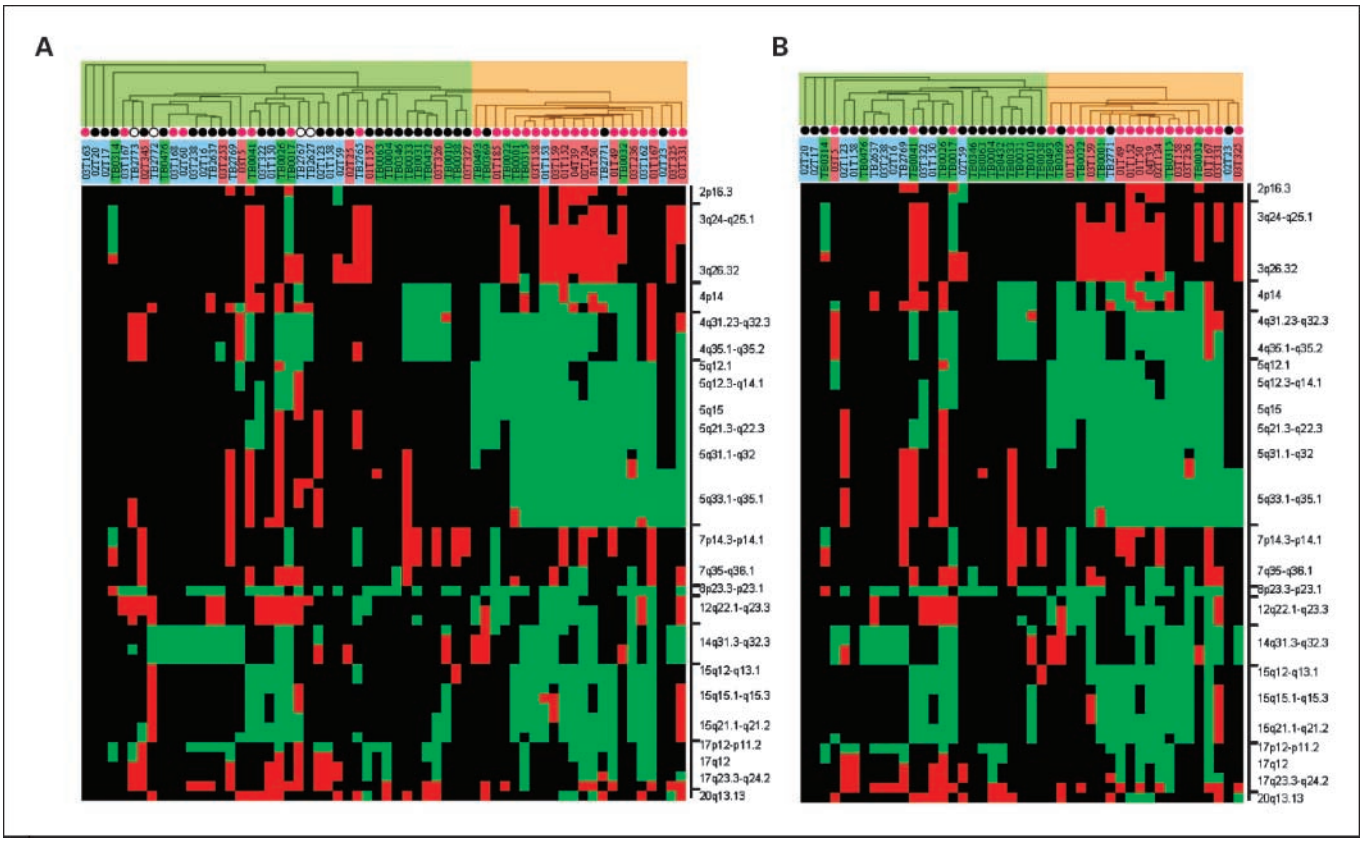

Fig. 3. *A,* unsupervised clustering of all BRCA1 (*red*), BRCA2 (*blue*), and sporadic (*green*) breast tumors of our collection using the regions reported as discriminative in the aCGH classifier by Jönsson et al. (20). Regions are listed according to chromosome number and are considered as gains (*red*), no change (*black*), or losses (*green*). Black circle, ER^+; red circle, ER^-; white circle, nonevaluated cases. *B,* unsupervised clustering of BRCA1 (*red*), BRCA2 (*blue*), and sporadic (*green*) breast tumors with BRCA1 ER^+ and BRCA2 ER^- tumors removed.

cancer, supports previous findings obtained using the cCGH technique and the same aCGH platform (20), and tests the discriminative regions defined in the latter study. To our knowledge, this is the first aCGH profiling of BRCAX-associated breast cancer samples and the second one of *BRCA1*- and *BRCA2*-associated tumors. Our results highlight the importance of ER status, suggesting that this feature should be considered when designing comparative studies of familial and sporadic breast cancer.

Genomic instability in familial and sporadic breast cancer. We have confirmed the high genomic instability in *BRCA1/2*-associated tumors that was previously observed in several articles using cCGH (13–16) and aCGH (20). The highest number of CNV was present in breast cancers associated with *BRCA1*, those related to *BRCA2* mutations were the second most unstable class of breast tumors, whereas sporadic cases showed more CNV than BRCAX-related breast cancers but less than those associated with *BRCA2* mutations (Supplementary Fig. S1A).

We distinguished common aberrations across all breast cancer groups, such as gains of 1q and 16p13.3 and losses of 8ptel-p12 and 16q, which are concordant with previous analyses by cCGH (15, 16, 33). These commonly altered regions could represent a set of shared aberrations that include important genes and characterize the breast cancer development in general.

Beyond these shared alterations, there were no specific genomic changes associated with any specific tumor class, although some recurrent aberrations were observed (Fig. 2). Thus, *BRCA1*-associated tumors were characterized by a high frequency of gains of 3q and 8q21.3-qtel and losses of 4q32.3-qtel and distinct regions of chromosome 5. *BRCA1*-associated tumors also had a significantly recurrent gain at 11q14.1 when compared with *BRCA2*-associated tumors (Fig. 1; Table 1). Some of these changes have been reported as discriminative in tumor class comparisons (13, 15, 20). On the other hand, *BRCA2*-associated tumors had frequent gains of 8q12.3-qtel (significant when compared with BRCAX) and losses of 11q23.1-qtel, 13q12.3-q21.33, and chromosome 22 (Fig. 1; Table 1). Gain of 17q22-q24 and losses of chromosome 13 among others have been reported as frequent changes in *BRCA2*-associated tumors (13, 20). Here, we found both changes at a high frequency, and we were also able to refine both changes to 17q23-qtel and 13q12.3-q21.33. BRCAX tumors were characterized by the lowest overall frequency of genomic alterations, presumably due to the high number of grade 1 samples in the BRCAX-associated breast cancer set (14 of 31; see Supplementary Table S1). Finally, regions recurrently aberrant in sporadic samples were concordant with the ones previously reported (34, 35).

In addition, amplification target sites slightly differed between tumor classes (Table 1). Interestingly, whereas c-*MYC* is amplified in all groups suggesting an universal amplification target site, neither *BRCA1*- nor *BRCA2*-associated tumors presented amplification at the *ERBB2* locus, as previously

described (10, 20). *ERBB2* amplification seems to be a marker that can help in discriminating familial samples, in which mutation analysis of the BRCA genes is advisable, saving time and effort.

With these results, the aCGH patterns of familial breast cancer may assist in the diagnostics because there are differences between BRCA1 and BRCA2/BRCAX patients. However, our findings show some differences when compared with previous studies about the discriminative regions associated with familial and sporadic cancer. These variations are largely attributable to differences in sample size or the type of *BRCA1/2* mutation of the set of familial cancer samples, but we propose an additional role of ER status. Whereas previous studies mainly studied BRCA1/ER$^-$ and BRCA2/ER$^+$ tumors, we were able to study a set of *BRCA1*- and *BRCA2*-associated breast cancers that contained both ER$^+$ and ER$^-$ tumors.

ER status modulates the genomic changes in the tumor. Steroid receptor status is one of the main differentiating features of sporadic breast cancer, as has been shown in gene expression studies (36) and in aCGH profiles (34, 35, 37). With regard to familial breast cancer, *BRCA1*-associated tumors mainly are associated with ER negativity, and for this reason, most of studies on BRCA1 tumors only include ER$^-$ *BRCA1*-associated breast cancer samples. Examples are the study by Hedenfalk et al. (38) using expression arrays and more recently the study by Jönsson et al. (20) using aCGH. It has been suggested that the ER status may have confounded the findings in these studies (39, 40). When we examined our series using Jönsson's discriminative regions, we found one cluster mainly composed of *BRCA1*-associated cases and another one consisting of *BRCA2*-associated and sporadic cases, although many outliers were present (Fig. 3A). The reason could be that

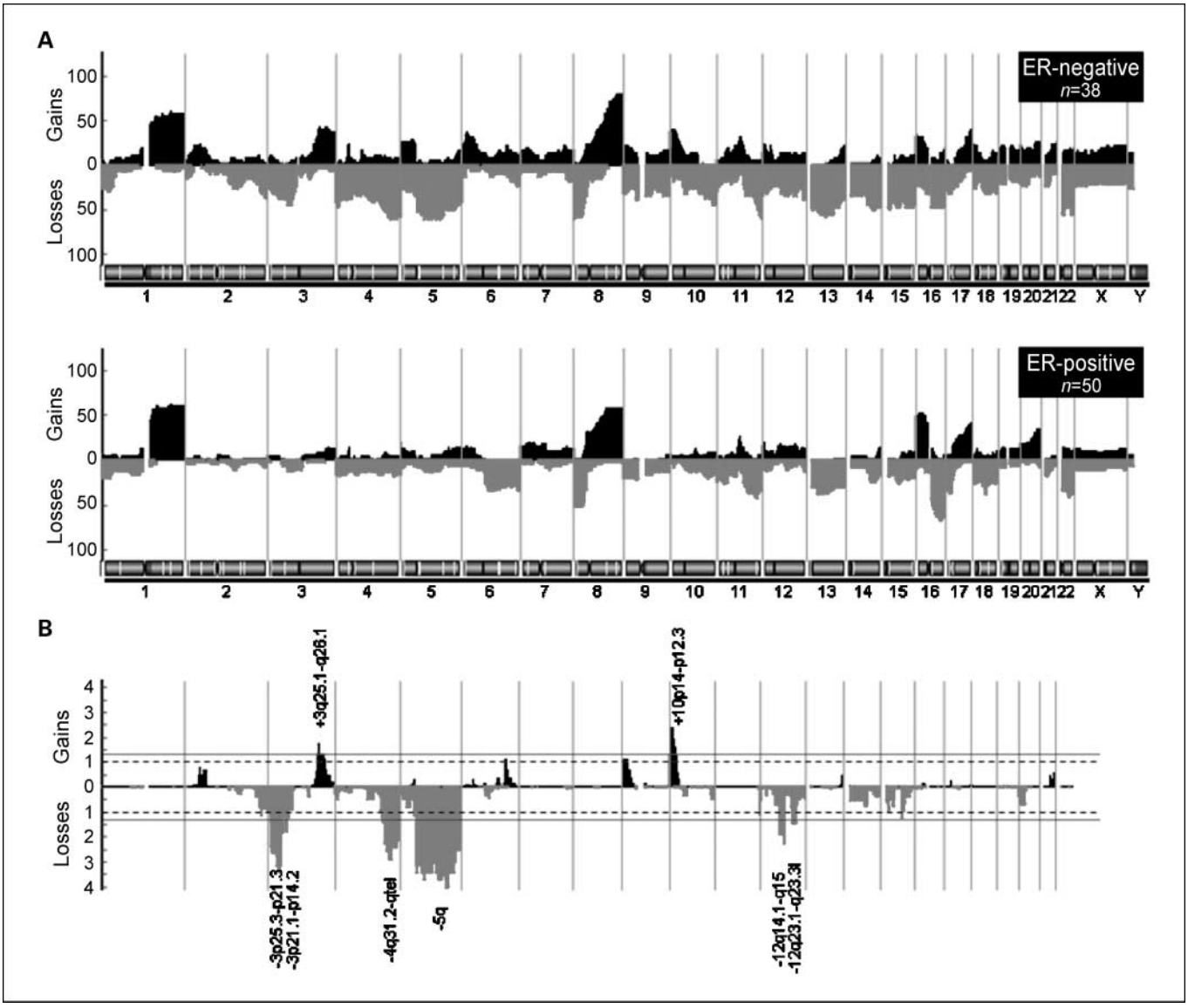

Fig. 4. *A,* overall genomic aberration frequencies of all tumors classified according to ER status. Black, frequency of gains; gray, frequency of losses. *B,* representation of the negative log of the adjusted *P* values, obtained from Fisher's exact test when comparing the gain (*top*) and loss (*bottom*) frequencies among ER$^-$ versus ER$^+$ tumors. Dashed lines, adjusted *P* value = 0.1 [-log (adjusted *P*) = 1]; gray lines, adjusted *P* value = 0.05 [-log (adjusted *P*) = 1.3]. Those aberrations that are statistically significant (*P* = 0.05, Fisher's exact test) are shown.

Jönsson's discriminative regions were found only comparing BRCA1 ER^-, BRCA2 ER^+, and sporadic tumors with either status, whereas our series included BRCA1 and BRCA2 tumors with a mix of ER^- and ER^+ status. In fact, after removing BRCA1 ER^+ and BRCA2 ER^- tumors from our series, we obtained one cluster composed of ER^- tumors (BRCA1 and sporadic) and a second cluster mainly formed by ER^+ tumors (BRCA2 and sporadic), the number of BRCA outliers being clearly reduced (Fig. 3B). Both ER clusters present obvious genomic differences from each other. These results suggest that ER status is an important marker of changes in the tumor genome, independent of the underlying mutation status.

When we grouped all tumors, including BRCAX samples, according to their ER status, the higher genomic instability of ER^- tumors was clearly shown not only when all cases were grouped together (Supplementary Fig. S1B) but also within each group defined by BRCA mutation status (Supplementary Fig. S1C). Several genomic aberrations were recurrently present in ER^- tumors, such as -3p25.3-p21.3, -3p21.1-p14.2, +3q25.1-q26.1, -4q31.2-qtel, -5q, +10p14-p12.3, -12q14-q15, and -12q23.1-q23.31, which significantly discriminated between both ER tumor groups (Fig. 4B). Most of these aberrations are concordant with those previously reported in aCGH studies of sporadic breast cancer (34, 35, 37), and some were also included in Jönsson's discriminative regions (20). In fact, losses of loci at 4q and 5q were reported as frequent in *BRCA1*-related breast cancers but also in ER^- *BRCA2*-associated breast tumors and ER^- sporadic samples (41, 42). On the other hand, ER^+ tumors showed a lower level of genomic instability and a trend to present alterations at chromosome 16 (+16p and -16q), which are classic features of ER^+ and low-grade tumors (35, 43, 44). Interestingly, absence of expression of ER seems to be due to a mechanism independent of copy number losses at the *ESR* locus, given that ER^+ tumors presented a higher deletion frequency at this locus than ER^- tumors (Fig. 4A). About high-level DNA amplifications, ER^- tumors had higher frequencies of these aberrations than ER^+ tumors. We propose here the significantly recurrent amplification at 13q34 in ER^- tumors as a candidate aberration for further characterization. The same differences between both tumor groups (ER^+/ER^-) about genomic instability and aberrant regions were also present when tumors were subdivided by BRCA mutation status (Supplementary Fig. S2), which emphasizes the role of the ER status independent of the breast tumor class. In this sense, comparisons between tumor classes with the same ER status could elucidate aberrations specifically associated with a breast tumor class. However, a larger amount of samples would be needed.

In summary, we present a genomic characterization of familial and sporadic breast cancer samples using aCGH. We confirm the higher genomic instability of BRCA1/2 tumors and describe the common existence of aberrations that could represent the set of genomic abnormalities characteristic in breast cancer development. We also report a set of altered regions that discriminate between tumor groups but are not specific for only one tumor class. Our findings suggest a critical role for the ER status as a marker of the genomic changes present in a tumor. The patterns of genomic changes among familial and sporadic breast cancers are strikingly similar, and differences are mainly determined by the ER status rather than by the BRCA mutation status as is generally accepted. Thus, ER negativity might allow or involve a set of genomic alterations different from those in tumors expressing ER. These aberrant regions may contain interesting genes that determine the higher aggressiveness of the ER^- tumors and, hence, should be analyzed in detail, whereas the BRCA gene mutation status (mainly BRCA1) would contribute to the genomic profile of abnormalities by increasing or modulating the genomic instability.

Acknowledgments

We thank the Spanish National Tumor Bank Network and Luz Álvarez and Miguel Urioste (Familial Cancer Unit) for the collection of the samples, the Immunohistological Unit and Carmen Martin (Molecular Cytogenetics Group, Spanish National Cancer Center) for their technical support, and Angelica C. Medina (Medical Genetics Division, University of Pennsylvania) for her technical assistance.

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Oncogene (2007), 1–11

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ORIGINAL ARTICLE

Distinct genomic aberration patterns are found in familial breast cancer associated with different immunohistochemical subtypes

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Five breast cancer subtypes have been described in sporadic breast cancer (SBC) using expression arrays: basal-like, ERBB2, normal breast-like, luminal A and B. These molecular subtypes show different genomic aberration patterns (GAPs). Recently, our group described these breast cancer subtypes in 50 non-*BRCA1/2* familial tumors using immunohistochemistry assays. We extended this study to the other classes of familial breast cancer (FBC), including 62 tumors (18 *BRCA1*, 16 *BRCA2* and 28 non-*BRCA1/2*), with the same panel of 25 immunohistochemical (IHC) markers and histological grade obtaining a similar classification. We combined these data with results generated by a 1 Mb BAC array-based CGH study to evaluate the genomic aberrations of each group. We found that *BRCA1*-related tumors are preferentially basal-like, whereas non-*BRCA1/2* familial tumors are mainly luminal A subtype. We described distinct GAPs related to each IHC subtype. Basal tumors had a greater number of gains/losses, while luminal B tumors had more high-level DNA amplifications. Our data are similar to those obtained in SBC studies, highlighting the existence of distinct genetic pathways of tumor evolution, common to both SBC and FBC.
Oncogene advance online publication, 10 December 2007; doi:10.1038/sj.onc.1210975

Keywords: hereditary breast cancer; BRCA1; BRCA2; BRCAX; array-CGH; breast cancer subtypes

Introduction

Human breast cancer is a heterogeneous disease encompassing different pathological entities and a range of clinical behavior. Studies in sporadic breast cancer (SBC) based on expression profiling reflect this heterogeneity (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003; Sotiriou *et al*., 2003). Five distinct SBC subtypes defined by different expression patterns and clinical outcomes have been reported: luminal A and B, ERBB2, basal-like and normal breast-like (Sorlie *et al*., 2003). Recently, two different studies have described distinct spectra of DNA copy number alterations associated with each SBC subtype. A higher number of gains/losses were associated with basal-like tumors, while high-level DNA amplification was more frequent in luminal-B subtype tumors (Bergamaschi *et al*., 2006; Chin *et al*., 2006).

Familial breast cancer (FBC) includes tumors from patients carrying mutations in the two known breast cancer susceptibility genes: *BRCA1* (Miki *et al*., 1994) and *BRCA2* (Wooster *et al*., 1995). However, most of FBC patients do not carry mutations in these genes, and are known as non-*BRCA1/2* or BRCAX cases. *BRCA1*-associated tumors can be differentiated from *BRCA2*, BRCAX and SBC based on their immunohistochemical (IHC) profiles (see reviews (Honrado *et al*., 2005a; Lacroix and Leclercq, 2005)). Most reports suggest that *BRCA1*-associated tumors have a basal-like phenotype because they share many expression, IHC and clinical features with basal-like cancers (see reviews (Tischkowitz and Foulkes, 2006; Turner and Reis-Filho, 2006)). On the other hand, *BRCA2*-associated tumors are only distinguished from BRCAX and SBC by the expression of DNA repair proteins such as RAD51 and CHEK2 (Honrado *et al*., 2005b). The association of *BRCA2*-associated tumors with luminal A subtype has been suggested in a single expression study (Sorlie *et al*., 2003). Finally, recent IHC studies have underlined the heterogeneous of the BRCAX tumors, which resembles the one obtained in SBC studies (Oldenburg *et al*., 2006; Honrado *et al*., 2007).

Although the genomic characterization of *BRCA1*-, *BRCA2*- and BRCAX-associated tumors has been reported in different studies (Tirkkonen *et al*., 1997; Alvarez *et al*., 2005; Jonsson *et al*., 2005; Melchor *et al*., in press; van Beers *et al*., 2005), the possible correlation between the molecular subtypes of FBC and the

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Received 11 September 2007; revised 1 November 2007; accepted 8 November 2007

genomic aberrations has not been clarified yet. In the present study, we have classified 62 FBC samples by IHC analysis into five subgroups. We have demonstrated a correlation between IHC subtypes and specific Genomic Aberration Patterns (GAPs). Our results are very similar to those obtained previously in SBC, and support that breast cancer arises from several distinct biological mechanisms, which are common to both FBC and SBC.

Results

Data from 62 FBC samples (18 *BRCA1*-, 16 *BRCA2*- and 28 BRCAX-associated tumors) previously studied by IHC and aCGH have been correlated to establish their IHC subtypes and the pattern of genomic aberrations.

Unsupervised cluster analysis

We performed an unsupervised hierarchical cluster analysis with 25 IHC markers (Supplementary Table 1) and histological grade. The 62 tumor samples were classified into two main groups, associated with their estrogen receptor (ER) status. The ER-negative branch (Figure 1, left) included 20 tumors of grade 2 and 3 (brown and red squares, respectively), hormonal receptors negativity and overexpression of proteins that promote cell cycle progression and cell proliferation. The ER-positive branch involved 42 tumors mainly of grade 1 (green squares) or 2, hormonal receptors positivity and expression of proteins related to the inhibition of the cyclin-CDK complexes, and luminal epithelial proteins (cytokeratin 8 (CK8)) (Figure 1, right).

The ER-negative branch can be split into two subgroups: one characterized by ERBB2 overexpression (4 tumors), and a second subgroup (16 tumors) defined by overexpression of basal markers, such as, cytokeratin 5 (CK5), vimentin, survivin and epidermal growth factor receptor (EGFR). The subgroup associated with ERBB2 overexpression was composed exclusively of BRCAX tumors, while the basal-like subgroup included mainly *BRCA1*-associated tumors (11 of 16 tumors).

Three subgroups were found within the ER-positive branch. One of the subgroups (brown branch in Figure 1) contained 16 tumors that are mostly grade 1, and overexpress hormonal receptors, CK8, BCL2 and proteins that inhibit cell cycle progression (for example, p27 and p16). Of note, most of these tumors were BRCAX samples (13 of 16). A total of 17 tumors composed the second subgroup (purple branch in Figure 1). These samples were characterized by a higher grade than the previous group, low or lack of expression of hormonal receptors as compared with the other groups and overexpression of other proteins such as cyclin A (related to the cell cycle progression) and TOPOII (related to cell growth). A mixture of the three FBC groups was found in this subgroup (Figure 1). We termed these two subgroups as luminal A and B, respectively, according to the parameters (such as grade and differential expression of TOPOII) that have been used previously to discriminate between them (Sorlie *et al*., 2001; Sotiriou *et al*., 2003). Finally, a group of nine tumors were described (Figure 1, green branch), which had luminal features (positive for hormonal receptors and CK8), high grade and overexpression of CHEK2 and survivin. This group was composed mainly of *BRCA2*-associated tumors (5 of 9 samples). We called this group as 'unclassified' tumors, since the features did not match with those previously established for 'normal breast-like' or the other tumor groups.

A summary of the FBC distribution in the different IHC subtypes is shown in Table 1. Most of *BRCA1*-associated tumors show a basal-like phenotype, while

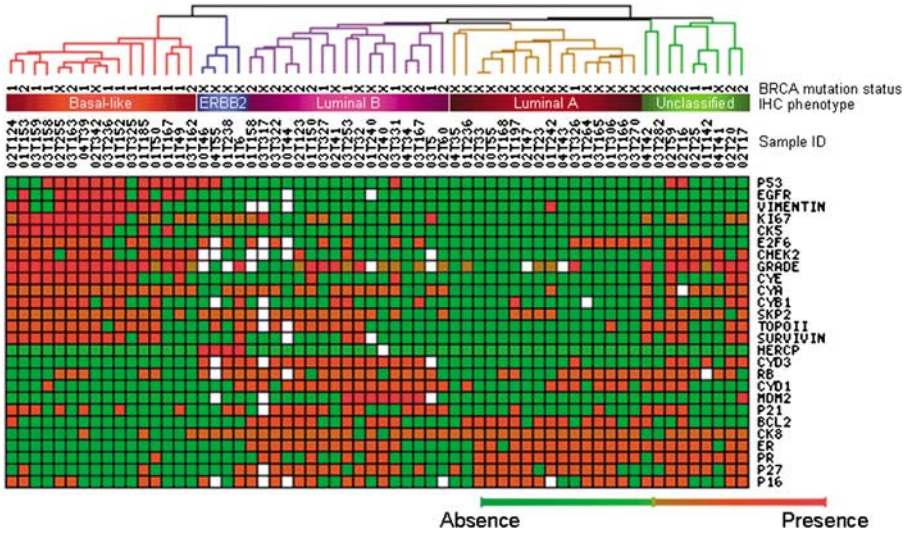

Figure 1 Unsupervised hierarchical clustering of 62 FBC samples. White squares correspond to data not available. The percentage of positive cells for each immunohistochemical marker is represented as a range of color between the most green (lowest percentage) and the most red (highest percentage). Intermediate colors represent percentages between the lowest and the highest.

Table 1 Immunophenotype distribution of the FBC tumors based on the 25 IHC markers and histological grade

	Basal-like	*ERBB2*	*Luminal B*	*Luminal A*	*Unclassified*
BRCA1 ($n=18$)	**11 (61.1%)**[a]	0	4 (22.2%)	1 (5.6%)	2 (11.1%)
BRCA2 ($n=16$)	3 (18.8%)	0	**6 (37.5%)**	2 (12.5%)	**5 (31.2%)**
BRCAX ($n=28$)	2 (7.1%)	4 (14.3%)	7 (25.0%)	**13 (46.5%)**[b]	2 (7.1%)
Total ($n=62$)	16 (25.8%)	4 (6.5%)	17 (27.4%)	16 (25.8%)	9 (14.5%)

Abbreviations: FBC, familial breast cancer; IHC, immunohistochemical. Bold values indicate the BRCA-class that presents the highest percentage of a concrete immunophenotype. [a]Significant differences in the Fisher's exact test ($P \leqslant 0.05$) when comparing basal-like distribution of *BRCA1* versus *BRCA2*, and *BRCA1* versus BRCAX. [b]Significant differences in the Fisher's exact test ($P \leqslant 0.05$) when comparing luminal A distribution of BRCAX versus *BRCA1* and BRCAX versus *BRCA2*.

BRCA2-associated tumors are mainly found in the luminal B or unclassified subtypes. The ERBB2 subtype was composed entirely of BRCAX samples, although most of BRCAX malignancies had a luminal A phenotype. IHC characteristics of the different subtypes and statistical comparisons are shown in Table 2.

Genomic characterization of each FBC subtype

Classifying the FBC tumors in five subtypes according to the IHC clusters, we collected the array-CGH data of these samples (Melchor *et al*., in press), and assessed their copy number of genomic aberrations (CNA). Basal breast tumors had the highest mean of genomic changes (30.75 ± 3.0 CNA), while luminal A tumors had the lowest mean number of CNA (10.87 ± 1.9). Luminal B, ERBB2 and unclassified tumors had 20.00 ± 2.7, 14.75 ± 6.2 and 14.44 ± 3.7 CNA, respectively (Figure 2). Differences in the amount of CNA were statistically significant ($P<0.05$, Mann–Whitney *U*-test) when comparing basal breast tumors with any of the other cancer subtypes.

Next, we plotted the GAPs of each FBC subtype (Figure 3). The most recurrent aberrations (frequency over 50%) in luminal A subtype were gains at 1q and 16p, and losses at 11q23 and 16q. Luminal B tumors exhibited as recurrent aberrations (over 50%): −8ptel-p12, +8q21-qtel, −11q23.3-qtel, −14q31, +16p, −16q, +20q13.13-qtel, −22q. Given that the ERBB2 subtype was composed of only four tumors, the GAP was not informative, though all cases had gain at 17q12 (*ERBB2* locus). Basal breast tumors clearly presented an unstable GAP with many aberrations with a frequency over 50%, such as, −3p21-p13, +3q25.1-q26, −4p, −4q22.1-qtel, −5q, −8ptel-p12, +8q22.1-qtel, among others. Finally, unclassified tumors showed an intermediate level of genome instability as compared to luminal A and B tumors. The only two genomic aberrations with a frequency higher than 50% in unclassified tumors were gains at 1q32.2 and 8q21.12-qtel. When we compared the aberration frequencies between the subtypes as determined by IHC, significant differences were only found when compared basal versus non-basal breast tumors (Supplementary Figure 1).

High-level DNA amplifications were more frequently found in luminal B, basal-like and ERBB2 tumors than in luminal A cancers ($P=0.021$, 0.036 and 0.042, respectively) (Figure 4a). Some regions of high-level DNA amplification tend to be subtype specific such as 20q13 in luminal B tumors, 6p22 and 13q34 in basal-like, and as expected 17q12 in ERBB2 tumors (Figure 4b).

Discussion

We have shown that FBC can be grouped in the different breast cancer subtypes described previously in SBC using IHC (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003; Sotiriou *et al*., 2003). In addition, we have performed a complete genomic characterization of the subtypes. Differences in the levels of genomic instability, GAPs, and high-level DNA amplification target regions were found associated with FBC subtypes.

Common breast cancer heterogeneity and association of BRCA *status with breast cancer subtype*

We have previously shown the heterogeneity present in BRCAX and SBC tumors using 25 IHC markers and grade (Honrado *et al*., 2007). In the current work, we extended the study to the other classes of FBC, including 62 cancers (18 *BRCA1*-, 16 *BRCA2*- and 28 BRCAX-associated), and found similar results: five FBC subtypes were established using unsupervised cluster analysis of IHC of multiple proteins and grade (Figure 1). Each FBC subtype was associated with its own characteristic IHC features (Table 2). These subtypes were similar to those obtained in SBC using expression analysis: basal-like, ERBB2, luminal A and B (Perou *et al*., 2000; Sorlie *et al*., 2001). Although we were not able to distinguish a normal breast-like subtype, we identified a fifth group with intermediate characteristics between luminal A and B subtypes, which we titled the 'unclassified group'. Given its higher grade and over-expression of proliferation markers (cyclins, Ki-67 and so on), this subtype could be more aggressive than the luminal A subtype and thus, it could be analogous to the luminal C subtype named by Sorlie *et al*. (2001) or luminal-like 3 described by Sotiriou *et al*. (2003). These findings emphasize the existence of different breast tumor subtypes that represent distinct biological entities not only in SBC, but also in FBC.

Table 2 Immunohistochemical markers and statistical comparisons between the different IHC subtypes

	Basal-like (N = *16*)	*ERBB2* (N = *4*)	*Luminal B* (N = *17*)	*Luminal A* (N = *16*)	*Unclassified* (N = *9*)	P[1]	P[2]	P[3]	P[4]	P[5]
HERCP(+3)										
1–2+	16 (100.0)	0	16 (100.0)	16 (100.0)	9 (100.0)	NS	0.000	NS	NS	NS
3+	0	4 (100.0)	0	0	0					
ER										
Negative	16 (100.0)	4 (100.0)	4 (23.5)	3 (18.8)	1 (11.1)	0.000	0.037	0.047	0.019	0.033
Positive	0	0	13 (76.5)	13 (81.3)	8 (88.9)					
PR										
Negative	15 (93.8)	4 (100.0)	11 (64.7)	2 (12.5)	1 (11.1)	0.000	NS	NS	0.000	0.009
Positive	1 (6.2)	0	6 (35.3)	14 (87.5)	8 (88.9)					
P53										
Negative	4 (25.0)	2 (50.0)	16 (94.1)	16 (100.0)	7 (77.8)	0.000	NS	0.025	0.003	NS
Positive	12 (75.0)	2 (50.0)	1 (5.9)	0	2 (22.2)					
BCL2										
Negative	15 (93.8)	4 (100.0)	7 (41.2)	4 (25.0)	7 (77.8)	0.001	NS	0.086	0.002	NS
Positive	1 (6.3)	0	10 (58.8)	12 (75.0)	2 (22.2)					
Ki-67										
0–4%	2 (12.5)	1 (25.0)	10 (58.8)	16 (100.0)	4 (44.4)	0.000*	0.105*	NS*	0.000*	0.102*
5–24%	5 (31.3)	3 (75.0)	5 (29.4)	0	5 (55.6)					
25–100%	9 (56.3)	0	2 (11.8)	0	0					
EGFR										
Negative	6 (37.5)	4 (100.0)	15 (100.0)	16 (100.0)	9 (100.0)	0.000	NS	0.054	0.050	NS
Positive	10 (62.5)	0	0	0	0					
CK5										
Negative	5 (31.3)	4 (100.0)	17 (100.0)	16 (100.0)	9 (100.0)	0.000	NS	0.026	0.052	NS
Positive	11 (68.8)	0	0	0	0					
Vimentin										
Negative	5 (31.3)	4 (100.0)	14 (100.0)	15 (93.8)	9 (100.0)	0.000	NS	0.052	NS	NS
Positive	11 (68.8)	0	0	1 (6.3)	0					
Grade										
1	0	1 (50.0)	4 (28.6)	10 (71.4)	1 (11.1)	0.001*	NS*	0.066*	0.000*	NS*
2	2 (12.5)	0	6 (42.9)	3 (21.4)	1 (11.1)					
3	14 (87.5)	1 (50.0)	4 (28.6)	1 (7.1)	7 (77.8)					
CK8										
Negative	13 (81.3)	0	2 (11.8)	0	2 (22.2)	0.000	NS	NS	0.003	NS
Positive	3 (18.8)	4 (100.0)	15 (88.2)	16 (100.0)	7 (77.8)					
Cyclin D1										
Negative	15 (93.8)	3 (75.0)	1 (5.9)	9 (56.3)	5 (55.6)	0.000	NS	0.000	NS	NS
Positive	1 (6.3)	1 (25.0)	16 (94.1)	7 (43.8)	4 (44.4)					
Cyclin D3										
Negative	16 (100.0)	0	1 (6.7)	15 (93.8)	4 (44.4)	0.000	0.054	0.000	0.002	NS
Positive	0	3 (100.0)	14 (93.3)	1 (6.3)	5 (55.6)					
Cyclin E										
Negative	4 (25.0)	4 (100.0)	15 (88.2)	16 (100.0)	5 (55.6)	0.000	NS	NS	0.003	NS
Positive	12 (75.0)	0	2 (11.8)	0	4 (44.4)					
Cyclin A										
Negative	0	2 (50.0)	4 (23.5)	15 (93.8)	1 (12.5)	0.000	NS	NS	0.000	NS
Positive	16 (100.0)	2 (50.0)	13 (76.5)	1 (6.3)	7 (87.5)					
Cyclin B1										
Negative	6 (37.5)	2 (66.7)	13 (81.3)	14 (93.3)	5 (55.6)	0.004	NS	NS	0.023	NS
Positive	10 (62.5)	1 (33.3)	3 (18.8)	1 (6.7)	4 (44.4)					
P27										
Negative	14 (87.5)	3 (75.0)	4 (25.0)	4 (25.0)	3 (33.3)	0.000	NS	0.079	0.079	NS
Positive	2 (12.5)	1 (25.0)	12 (75.0)	12 (75.0)	6 (66.7)					

Table 2 (*Continued*)

	Basal-like (N = *16*)	*ERBB2* (N = *4*)	*Luminal B* (N = *17*)	*Luminal A* (N = *16*)	*Unclassified* (N = *9*)	P[1]	P[2]	P[3]	P[4]	P[5]
SKP2										
Negative	2 (12.5)	0	4 (25.0)	12 (75.0)	2 (22.2)	0.063	NS	NS	0.000	NS
Positive	14 (87.5)	4 (100.0)	12 (75.0)	4 (25.0)	7 (77.8)					
P16										
Negative	10 (62.5)	1 (33.3)	7 (50.0)	5 (33.3)	3 (33.3)	NS	NS	NS	NS	NS
Positive	6 (37.5)	2 (66.7)	7 (50.0)	10 (66.7)	6 (66.7)					
P21										
Negative	9 (56.3)	2 (50.0)	5 (31.3)	14 (87.5)	5 (55.6)	NS	NS	0.019	0.007	NS
Positive	7 (43.8)	2 (50.0)	11 (68.8)	2 (12.5)	4 (44.4)					
RB										
Negative	11 (68.8)	0	3 (18.8)	7 (43.8)	1 (12.5)	0.005	NS	NS	NS	NS
Positive	5 (31.3)	3 (100.0)	13 (81.3)	9 (56.3)	7 (87.5)					
MDM2										
Negative	16 (100.0)	3 (100.0)	8 (53.3)	16 (100.0)	8 (88.9)	0.093	NS	0.000	0.093	NS
Positive	0	0	7 (46.7)	0	1 (11.1)					
E2F6										
Negative	6 (37.5)	1 (33.3)	9 (60.0)	10 (62.5)	4 (44.4)	NS	NS	NS	NS	NS
Positive	10 (62.5)	2 (66.7)	6 (40.0)	6 (37.5)	5 (55.6)					
TOPOII										
Negative	4 (25.0)	2 (50.0)	7 (46.7)	15 (93.8)	3 (33.3)	0.019	NS	NS	0.000	NS
Positive	12 (75.0)	2 (50.0)	8 (53.3)	1 (6.3)	6 (66.7)					
CHEK2										
Negative	3 (18.8)	2 (100.0)	11 (84.6)	16 (100.0)	3 (33.3)	0.000	NS	0.101	0.000	0.066
Positive	13 (81.3)	0	2 (15.4)	0	6 (66.7)					
Survivin										
Negative	4 (25.0)	3 (75.0)	13 (86.7)	16 (100.0)	4 (44.4)	0.000	NS	0.067	0.001	NS
Positive	12 (75.0)	1 (25.0)	2 (13.3)	0	5 (55.6)					

Abbreviations: EFGR, epidermal growth factor receptor; ER, estrogen receptor; IHC, immunohistochemical; NS, not significant; PR, progesterone receptor. *P*-values from the Fisher's exact test performed when compared one IHC subtype versus the rest: P^1, basal-like versus nonbasal like tumors; P^2, ERBB2 versus nonERBB2 tumors; P^3, luminal B versus nonluminal B tumors; P^4, luminal A versus nonluminal A tumors; P^5, unclassified versus non-unclassified tumors. (*) χ^2-test was used for Ki-67 and grade. *P*-values $\leqslant 0.10$ are represented; *P*-values $\leqslant 0.05$ were considered as significant; NS, not-significant *P*-values.

The proportion of these subtypes in FBC is not the same as in SBC (Sorlie *et al*., 2001) or BRCAX samples (Oldenburg *et al*., 2006; Honrado *et al*., 2007). In the present study, 26% of FBC had a basal-like IHC phenotype compared to 15% in SBC and BRCAX (Sorlie *et al*., 2001; Oldenburg *et al*., 2006) (Table 1). This difference can be attributable to the presence of *BRCA1*-associated tumors in our series, which are prone to have a basal phenotype (Sorlie *et al*., 2003; Tischkowitz and Foulkes, 2006; Turner and Reis-Filho, 2006). In our sample set, 61% of *BRCA1*-associated tumors comprised a significant proportion of the basal-like IHC phenotype. Nineteen percent of *BRCA2*-associated tumors, and 7% of BRCAX samples were also found to be basal-like (Table 1). All BRCAX cancers were studied previously for hypermethylation of the *BRCA1* gene promoter and loss of heterozygosity; interestingly, the BRCAX samples that had basal-like phenotype showed biallelic inactivation of the *BRCA1* gene (Honrado *et al*., 2007). This model of carcinogenesis in the BRCAX tumors is in agreement with the low level of *BRCA1* mRNA expression reported in basal-like cancers (Staff *et al*., 2003; Wei *et al*., 2005; Turner *et al*., 2006). On the other hand, the ERBB2 subtype comprised only of BRCAX tumors (four cases, ~14%) (Table 1). This finding is in concordance with the low incidence of *ERBB2* amplification in *BRCA1/2* mutation carriers described before (Grushko *et al*., 2002; Lakhani *et al*., 2002; Palacios *et al*., 2003; Adem *et al*., 2004). A significant association was found between most of BRCAX samples (45%) and luminal A phenotype, as seen in previous analyses (Oldenburg *et al*., 2006; Honrado *et al*., 2007). Finally, *BRCA2*- and non-basal *BRCA1*-associated tumors were mainly related to the luminal B phenotype (37 and 22%, respectively). *BRCA2*-associated tumors also comprised an important proportion of the unclassified samples (Table 1). In contrast, Sorlie *et al*. (2003) linked *BRCA2*-associated

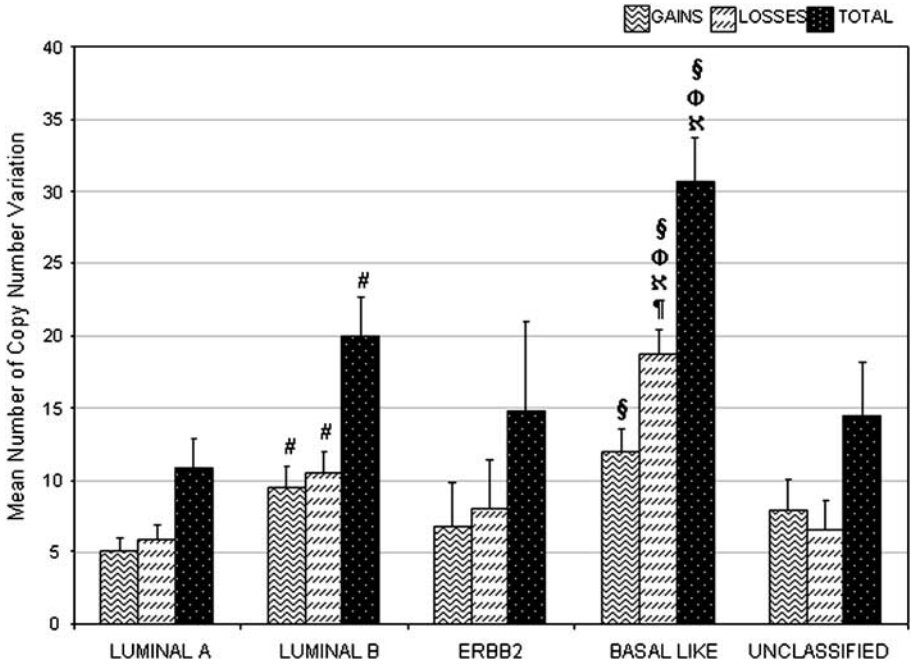

Figure 2 Mean number of genomic alterations. Significant differences (*P*-value <0.05 in Mann–Whitney *U*-Test) are shown in different characters depending on the comparison: basal-like versus luminal A (§), luminal B (Φ), unclassified (ϰ) ERBB2 (¶) and (#) luminal B versus luminal A tumors.

tumors to the luminal A subtype. This discrepancy may be caused by the small sample size given that the authors studied only two samples while we have a larger cohort of 16 *BRCA2*-associated cancers.

Distinct genomic aberration patterns associated with each FBC subtype

Different genomic characteristics have been recently associated with each of the five subtypes of SBC (Bergamaschi *et al*., 2006; Chin *et al*., 2006). We have studied the genomic change features of the five FBC subtypes using array-CGH data from a previous analysis (Melchor *et al*., in press). Basal-like tumors showed the highest genomic instability (Figure 2), consistent with two previous studies in SBC (Bergamaschi *et al*., 2006; Chin *et al*., 2006). In contrast, luminal A tumors had the lowest number of genomic aberrations. Bergamaschi *et al*. (2006) found the lowest number in ERBB2 tumors, but the low number of ERBB2 samples in our study (four cases) did not allow us to draw any significant conclusions.

The FBC subtypes defined by IHC exhibited distinct GAP (Figure 3). Basal-like tumors showed a specific GAP with many altered chromosomal sites such as -3p, $+3$q, -4p, -4q, -5q, -8p, $+8$q and so on. Some of these aberrations (for example, -3p25, -4p, -4q22-q35.1, -5q and so on) were significantly associated with basal-like tumors when compared with non-basal tumors (Supplementary Figure 1). The close association between basal-like phenotype and *BRCA1*-associated tumors explains the similarities that are found when comparing the GAP from basal-like tumors and *BRCA1*-associated tumors (Jonsson *et al*., 2005). Luminal A tumors frequently have $+1$q, $+16$p, -11q23 and -16q; luminal B tumors show genomic aberrations of other regions such as -8p, $+8$q, $+20$q and -22q. These subtype-GAPs and the recurrent chromosomal aberrations within each subtype are in agreement with those previously reported in SBC (Supplementary Table 2) (Bergamaschi *et al*., 2006; Chin *et al*., 2006).

Differences in high-level DNA amplifications

High-level DNA amplifications were found more frequently in luminal B tumors than in the other tumor subtypes (Figure 4a). In addition, the regions targeted for amplification differed slightly between the IHC subtypes (Figure 4b). As expected, ERBB2 tumors had a frequent amplification at 17q12 and overexpression of ERBB2. Approximately 20% of the luminal B breast cancer subtype had amplification of regions, such as, 8p11-p12, 8q24, 11q13.3-q13.4, 17q25 and 20q13. Luminal A cancers had few high-level amplifications with the exception of frequent amplification at 11q13 (*CCND1* locus), which was also found in luminal B tumors. This finding could explain the CCND1 over-expression present in the luminal tumors (Figure 1), and it is in concordance with the studies that show a negative correlation between *CCND1* amplification and basal-like phenotype (Reis-Filho *et al*., 2006; Elsheikh *et al*., 2007). Finally, basal-like tumors have high-level DNA amplification frequently at 8q24, 12p13 and 13q34

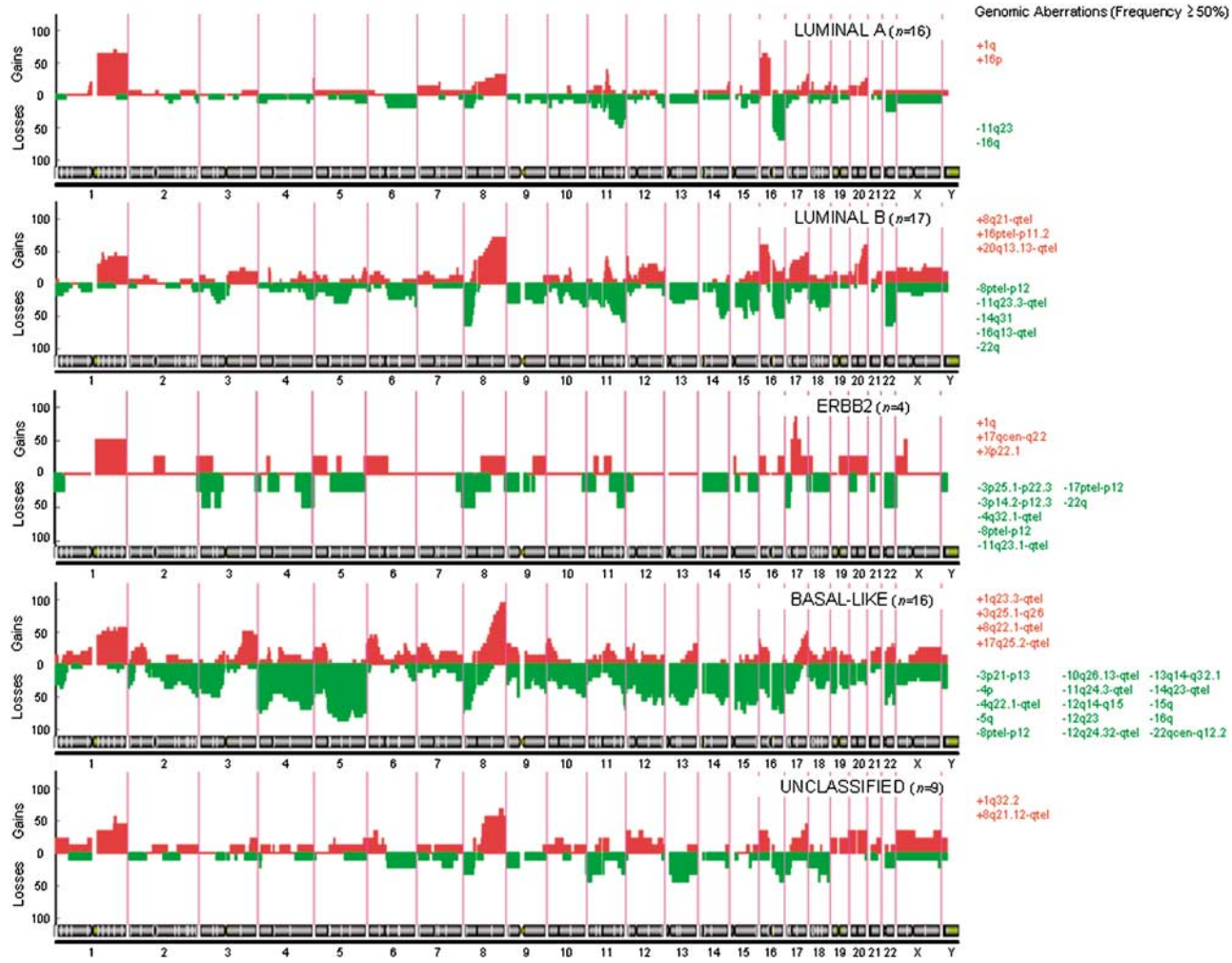

Figure 3 Genomic aberration patterns among the different IHC breast cancer subtypes. Red and green indicate frequencies of gains and losses, respectively. Genomic aberrations with a frequency over 50% in each IHC subtype are shown.

(Figure 4b). Most of these amplification sites are similar to the regions described in SBC subtypes (Bergamaschi *et al*., 2006; Chin *et al*., 2006) (Supplementary Table 2). Amplification of 11q13 appears recurrent in luminal cancers, whereas amplifications of 8p11-12 and 20q13 are more frequently found in the luminal B subtype, and amplification of 17q21 in the ERBB2 subtype. These common amplifications found in both FBC and SBC subtypes could represent targets for therapy, as has already been established with *ERBB2* and trastuzumab. A high frequency of amplification at 8q24 (*MYC* locus) was described in our basal-like FBC, an aberration less common in basal-like SBC (Chin *et al*., 2006; Rodriguez-Pinilla *et al*., 2006). Because Al-Kuraya *et al*. (2004) reported a greater frequency of *MYC* amplification in medullary carcinomas, a specific subtype of basal-like tumors that is very frequent in *BRCA1*-associated cancers (Lakhani *et al*., 1998), we checked whether that difference could be due to the presence of medullary carcinomas in our basal-like FBC. However, *MYC* amplification was present in 3 of 6 medullary FBC and 4 of 10 non-medullary basal-like FBC in our set (data not shown). A larger series of cases will be necessary to confirm or to rule out this association.

Conclusions

Our findings demonstrate that breast cancer can be subdivided into distinct subtypes independently whether the tumors are familial or sporadic. The FBC subtypes differed in terms of tumor histology, IHC portraits and genomic changes patterns (Table 3). A higher prevalence of basal-like phenotype is present in *BRCA1*-related tumors, while luminal A phenotype is recurrent in *BRCAX*-associated cancers. In addition, basal-like malignancies had more gains and losses than the other subtypes, while luminal B cancers showed more high-level DNA amplifications. These characteristics are similar to those recently described in SBC (Bergamaschi *et al*., 2006; Chin *et al*., 2006). These findings support the existence of distinct genetic pathways of tumor evolution, common to sporadic and FBC, which underlie the

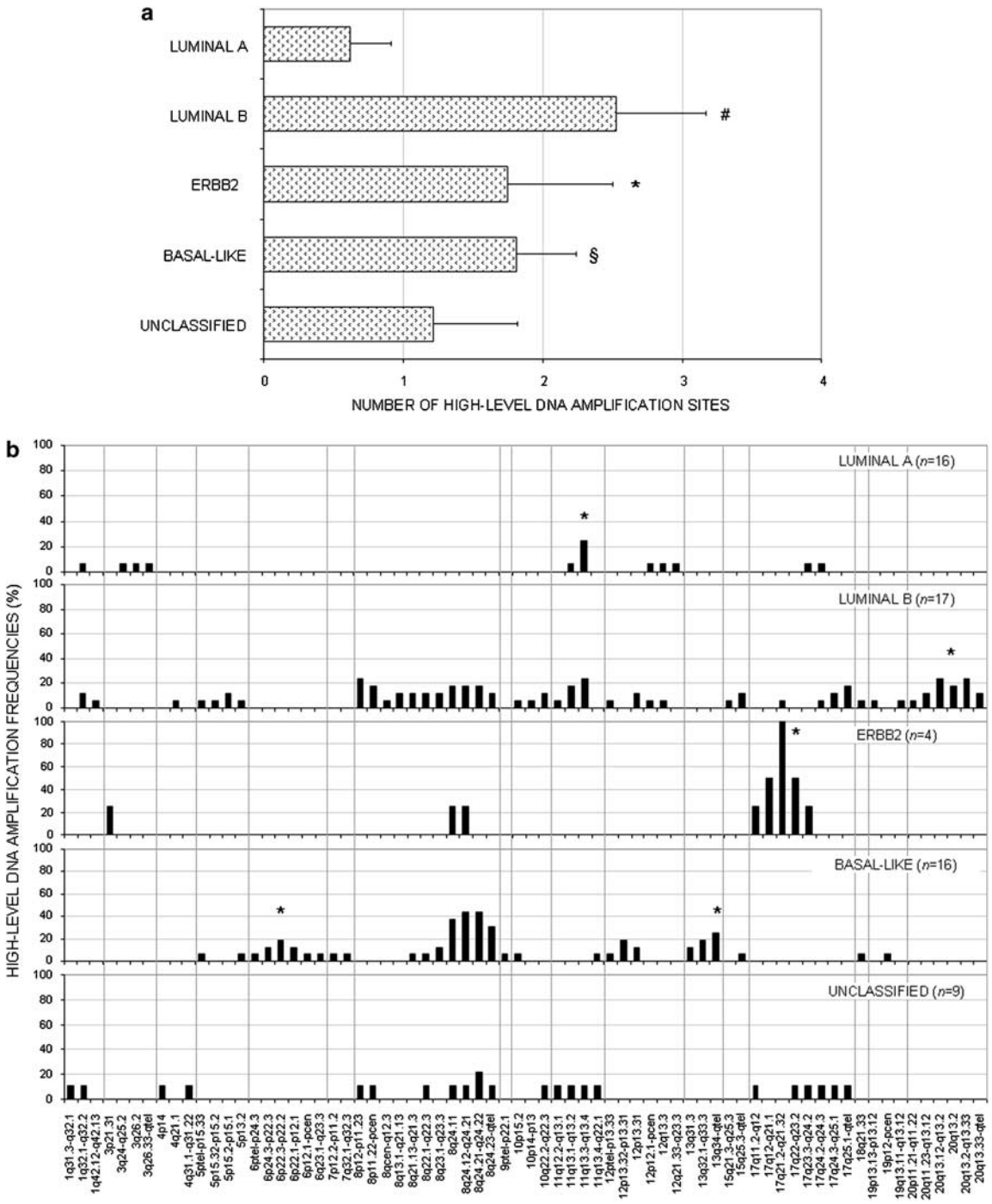

Figure 4 (**a**) Mean number of high-level DNA amplifications in each IHC breast cancer subtype. Significant differences (*P*-value <0.05 in Mann–Whitney *U*-Test) are shown in different characters depending on the comparison: luminal A versus luminal B (#), ERBB2 (*) and basal-like (§). (**b**) Frequency of high-level DNA amplification in each IHC breast cancer. Chromosomal sites of amplification are written in the *x* axis. Vertical gray lines separate chromosomes. Asterisks (*) point out specific amplification sites on each subtype.

pathogenesis of the different breast tumor subtypes and may explain their distinct biological behavior. Furthermore, we would postulate that gene-expression profiling, clinical presentation and response to therapy also differ in the five FBC IHC subtypes, as already reported in SBC subtypes (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003; Carey *et al*., 2006; Hu *et al*., 2006). Taking into account these differences, the BC subtypes should be studied as distinct entities to better describe their features, as has been done for basal-like tumors (Turner and Reis-Filho, 2006; Yehiely *et al*., 2006; Vincent-Salomon *et al*., 2007).

Table 3 Summary of features of IHC–FBC subtypes: basal-like, ERBB2, luminal A and B

	FBC class (%)							
IHC subtype	*BRCA1*	*BRCA2*	*BRCAX*	*Genomic instability*	*Recurrent gains*	*Recurrent losses*	*High-level DNA amplifications*	*Amplification targets*
Basal-like	68.75	18.75	12.50	High	1q, 3q, 8q, 17q	3p, 4p, 4q, 5q, 8p, 10q, 11q, 12q, 13q, 14q, 15q, 16q, 22q	Medium	6p22, 8q24, 13q34
ERBB2	0	0	100	Low	1q, 17q, Xp	3p, 4q, 8p, 11q	Medium	8q24, 17q12–q21
Luminal A	6.25	12.50	81.25	Low	1q, 16p	11q, 16q	Low	11q13
Luminal B	23.53	35.30	41.17	Medium	8q, 16p, 20q	8p, 11q, 14q, 16q, 22q	High	8p11–p12, 11q13, 20q13

Abbreviations: FBC, familial breast cancer; IHC, immunohistochemical. The first set of columns represents the distribution of every FBC class among one IHC phenotype. The second set of columns is the level of genomic instability (see Figure 2 for further details), with the recurrent gains and losses (see Figure 3 for further details of the chromosomal regions). The third set of columns shows the level of high-level DNA amplifications with the most recurrent targets (see Figure 4 for further details).

Materials and methods

Tumor samples and patients

We compiled 62 paraffin-embedded tumor tissues, which had been analysed previously both by IHC (Palacios *et al*., 2003, 2005; Honrado *et al*., 2005b) and aCGH (Melchor *et al*., in press). These breast cancer samples were selected from families with at least three women affected with breast and/or ovarian cancer and at least one of them diagnosed before 50 years of age, or from families with female breast and/or ovarian cancer and at least one case of male breast cancer. All cases were studied for mutations and large rearrangements in the *BRCA* genes using standard procedures (Osorio *et al*., 2000; Diez *et al*., 2003). A total of 18 cases had mutations in the *BRCA1* gene, 16 patients presented mutations in the *BRCA2* gene and 28 cases were negative for germ-line mutations in the *BRCA* genes (non-*BRCA1/2* or BRCAX).

Morphological evaluation, TMA construction and IHC studies

Histological sections from all 62 samples were reviewed by two pathologists (EH and JP). The Nottingham histological grading system was used to assess the grade of invasive ductal carcinomas.

Representative areas of the 62 tumors were carefully selected on H&E-stained sections and marked on individual paraffin blocks. Two tissue cores were obtained from each specimen and included in Tissue Micro-Arrays (TMA), whose characteristics have been previously published (Palacios *et al*., 2003, 2005; Honrado *et al*., 2005b).

Briefly, IHC assays were performed by the Envision method (Dako, Glostrup, Denmark) with a heat-induced antigen retrieval step. Sections from the tissue array were immersed in 10 mM boiling sodium citrate at pH 6.5 for 2 min in a pressure cooker. For the 25 antibodies used in this study, dilutions and suppliers are listed in Supplementary Table 1.

Between 150 and 200 cells per core were scored for the percentage of cells with positive nuclei or cytoplasm, depending upon the marker. We evaluated nuclear staining for ER, progesterone receptor, p53, Ki-67, cyclins D1, D3, E and A, p16, p27, p21, Skp2, retinoblastoma protein, E2F6, MDM2, topoisomerase IIα, survivin and CHEK2; evaluation of cytoplasmic staining was carried out for BCL2, vimentin, CK5/6, CK8 and cyclin B1, as described previously (Palacios *et al*., 2005). HER-2 expression was evaluated according to the four-category (0 to 3+) Dako system proposed for the evaluation of the HercepTest, and HER-2 expression of 3+ was the only value considered positive, as published earlier (Palacios *et al*., 2005).

Array comparative genomic hybridization analysis

Genomic DNA isolation from the 62 formalin-fixed paraffin-embedded (FFPE) tumors was performed as previously described (Melchor *et al*., in press). Briefly, two 30-μm sections were obtained from FFPE tumors, treated with xylene, incubated in Glycine Tris-EDTA (100 mM glycine, 10 mM Tris, pH 8.0, 1 mM EDTA) and NaSCN (1 M) and finally digested with proteinase K and purified with phenol chloroform. All sections were previously examined and dissected with a scalpel to ensure at least 70% content of tumor cells.

Comparative genomic hybridization was carried out onto the '1 Mb BAC' array platform developed at the University of Pennsylvania (Greshock *et al*., 2004). DNA probe labeling, aCGH protocol and array data analysis have been described previously (Melchor *et al*., in press). Briefly, in the array data analysis, aCGH normalization was carried using the DNMAD application (Vaquerizas *et al*., 2004). The normalized profiles were processed using the Binary Segmentation algorithm implemented in the Insilico CGH software (Vaquerizas *et al*., 2005). This algorithm defines genomic segments, which have an estimative copy number value in $\log_2$ ratio (that is, the median $\log_2$ ratio of the contained clones). Those segments with $\log_2$ ratio $\geqslant 0.1$ were considered as gains, while those with $\log_2$ ratio $\leqslant 0.1$ were categorized as losses. High-level DNA amplifications were considered when $\log_2$ ratio $\geqslant 0.4$.

Statistical analysis

Hierarchical unsupervised cluster analysis was performed by means of the UPGMA method using correlation distance and Euclidean distance between markers. The cluster was displayed using SOTAARRAY (Herrero *et al*., 2001) (software available at http://gepas.bioinfo.cipf.es). IHC results were represented by range of color from green to red, the lowest and the highest percentage of positive cells for each marker, respectively. Exceptions were grade that was scaled as 33% 'expressed' for grade 1 (green), 66% for grade 2 (brown) and 100% for grade 3 (red), and HER-2 that was scaled as 100% for positive (3+) (red) and 0% for negative (green) (Figure 1). Fisher's exact test was used to determine the differences in the expression of each marker between groups, except for Ki-67 and grade, which were calculated using χ^2-square test. The statistical software SPSS for Windows (SPSS Inc., Chicago, IL) was used for this analysis.

Regarding the array-CGH data, we used a non-parametric Mann–Whitney *U*-test to compare the mean number of genomic alterations among the different established groups. The SPSS software was used for these comparisons. For the analysis of differences in the aberration frequency of specific chromosomal regions, we used the Stat POMELO tool (http://pomelo.bioinfo.cnio.es) (Herrero *et al*., 2003), applied Fisher's

exact test and adjusted *P*-values for multiple testing using the FDR approach (a *P*-value <0.05 was considered significant).

Abbreviations

aCGH, array-based comparative genomic hybridization; CNA, copy number of genomic aberrations; ER, estrogen receptor; FBC, familial breast cancer; GAP, genomic aberration pattern; IHC, immunohistochemical; SBC, sporadic breast cancer.

Acknowledgements

We thank the Spanish National Tumor Bank Network, and Luz Álvarez and Miguel Urioste from the Familial Cancer Unit for the collection of the samples. For their technical assistance, we thank the Immunohistological Unit, and Carmen Martin and Juan C Cigudosa from the Molecular Cytogenetics Group, at the Spanish National Cancer Center and C Angelica Medina from Medical Genetics Division of the University of Pennsylvania. We also thank Susanna Leskelä for English assistance. *LM Grant sponsor*: Spanish Ministry of Education and Science FPU AP-2004-0448. Short stays sponsorships: ICRETT/05/063 and EMBO Short-Term Fellowship ASTF 162-05. MJG was supported by the Fundación Científica de la Asociación Española contra el Cáncer. This study has been partially funded by projects SAF03-02497 and SAF 06-06149 from the Spanish Ministry of Education and Science; and by a grant from Breast Cancer Research Foundation (BCRF) to KLN.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).